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THE PROFILE OF STEROID HORMONES, PROSTAGLANDINS AND CYTOKINES DURING TERM AND ETHANOL-INDUCED PRETERM PARTURITION IN THE MOUSE

by

Deborah Hong-Wei Sung

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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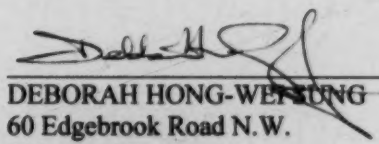

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To Mom, Dad, Derek and Daryl

for your unfailing love and support

ABSTRACT

Preterm birth continues to be a major problem in obstetrics. In mice, acute ethanol exposure late in gestation induces preterm birth, but the underlying mechanisms have not been elucidated. This study was designed to establish radioimmunoassays, ELISAs and bioassays in our laboratory, and to examine the chronological changes in various mediators from late gestation until the time of birth (delivery of the first pup), either at term or preterm. Placental and amniotic fluid interleukin-10 and interferon-γ were not detectable. However, term birth was associated with significant ($p \leq 0.05$) increases in plasma 17β-estradiol, the plasma estrogen to progesterone ratio, uterine and placental prostaglandin $F_{2\alpha}$, uterine prostaglandin E_2 and placental tumour necrosis factor levels. In contrast to term, only the estrogen to progesterone ratio and uterine prostaglandin E_2 levels were elevated at the time of preterm birth. These data suggest that term birth and ethanol-induced preterm birth are the consequence of different underlying mechanisms.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin
ANOVA	analysis of variance
ATPase	adenosine triphosphatase
Bmax	maximum binding
Ca^{2+}	calcium ion
cAMP	cyclic adenosine monophosphate
CAPs	contraction associated proteins
CMI	cell-mediated immunity
COV	coefficient of variation
CPM	counts per minute
CTL	cytotoxic T lymphocytes
DHEA/DHEAS	dehydroepiandrosterone (sulfate)
DHP	dihydroprogesterone
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EP	prostaglandin E receptor
E:P	estrogen to progesterone ratio
E_2:P	estradiol to progesterone ratio

E_3:P	estriol to progesterone ratio
ER	estrogen receptor
ERE	estrogen response elements
FBS	fetal bovine serum
FP	prostaglandin F receptor
FSH	follicle-stimulating hormone
GD	gestational day
GM-CSF	granulocyte-monocyte colony-stimulating factor
hCG	human chorionic gonadotropin
HPA	hypothalamo-pituitary-adrenal axis
HSD	hydroxysteroid dehydrogenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's Modified Dulbecco's Medium
kDa	kiloDaltons
LH	luteinizing hormone
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLCK	myosin light chain kinase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-κB
NK cell	natural killer cell

NSAID	non-steroidal anti-inflammatory drug
NSB	non-specific binding
P450scc	P450 side chain cleavage complex
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGDH	15-hydroxyprostaglandin dehydrogenase
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFM	13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$
PGHS	prostaglandin endoperoxide H synthase
PKA	protein kinase A
PLA_2	phospholipase A_2
PLC	phospholipase C
PR	progesterone receptor
PRE	progesterone response elements
ra	receptor antagonist
RIA	radioimmunoassay
RU486	17β-hydroxy-11β-(4-methylaminophenol)-17α-(prop-1-ynyl) estra-4,9-diene-3-one
SD	standard deviation
SEM	standard error of the mean
T	total binding
TGF	transforming growth factor

TH cells	helper T lymphocytes
TNF(R)	tumour necrosis factor (receptor)
v/v	volume per volume
w/v	weight per volume

CHAPTER I. BACKGROUND

A. INTRODUCTION

Parturition is the exquisitely orchestrated transition from pregnancy maintenance to pregnancy termination. Conceptually, it is beneficial to think of parturition in four over-lapping phases (Casey and MacDonald, 1993). The first phase is a period of uterine *quiescence* during which the spontaneous contractile activity of the uterus is minimal with only occasional uncoordinated contractures and the uterus is unresponsive to stimulation by contractile agents. For the majority of pregnancy, this phase predominates to accommodate the growth and development of the fetus and is characterized by high levels of mediators such as progesterone, nitric oxide and cytokines. Subsequently, a phase of uterine *activation* occurs in order to enhance the ability of the myometrium to respond to stimulation by contractile agents and to contract in a co-ordinated, synchronous manner. In this phase, there is an up-regulation of contraction associated proteins (CAPs) under the influence of estrogens, cytokines and mechanical stretch of the uterus (Challis and Lye, 1994). The CAPs include connexin-43, the major gap junction protein, as well as receptors for prostaglandins and oxytocin and the enzymes responsible for the synthesis of uterotonins (Challis *et al.*, 1997). The process of cervical ripening is also initiated during this phase. Uterine *stimulation* is the next phase which ensues and involves rupture of the fetal membranes, co-ordinated, rhythmic contractions of the myometrium and the birth of the baby as a consequence of stimulation by endogenous prostaglandins and oxytocin to produce contractions (Olson *et al.*, 1995). The final phase is a period of uterine *involution* during which the separation and

expulsion of the placenta occurs and the non-pregnant uterine menstrual cycle is re-established.

This thesis will focus on the events leading up to parturition in the mouse with specific emphasis on the role of steroid hormones, prostaglandins and cytokines. However, as a consequence of the relative scarcity of literature on parturition in the mouse which will be addressed at the end of this chapter, the more extensive literature in humans and other animal models will be reviewed in order to provide a more comprehensive concept of the physiology of parturition.

B. HUMAN PARTURITION AT TERM

Parturition normally occurs following 37-41 weeks of completed gestation when the start of gestation is defined as the first day of the last normal menstrual period (World Health Organization, 1977).

1. COMPONENTS OF PARTURITION

In order for a fetus to be successfully delivered, the physiological events which are essential to parturition must occur. These events include ripening of the cervix, rupture of the fetal membranes and, finally, expulsion of the fetus through rhythmic, co-ordinated contractions of the uterine myometrium.

a. Cervical Ripening

For the majority of pregnancy, the cervix is rigid and firm thereby conferring a functional seal to the uterus and its contents and allowing gestation to progress. Towards the end of pregnancy, the extracellular matrix of the cervix undergoes a dynamic re-organization to transform the cervix into a soft, distensible organ capable of allowing the passage of the fetus from an intrauterine to an extrauterine existence.

The cervix is composed primarily of the extracellular matrix as well as fibroblasts and a small proportion of smooth muscle. Water and collagen (protein) are the main constituents of the extracellular matrix, along with proteoglycans (dermatan sulfate, hyaluronate), glycoproteins (fibronectin) and a small amount of elastin. Dermatan sulfate contributes to collagen fibre stability, hyaluronate possesses a high water-binding capacity, and fibronectin binds to collagen and blocks the access of enzymes to the cleavage site of collagen. The process of cervical ripening begins about four weeks before birth and is likened to an inflammatory reaction (Liggins, 1978 and 1981). The distensibility of the cervix is increased through an increase in water content (increased hyaluronate levels), a destabilization of the collagen fibril network (decreased dermatan sulfate levels) and an increase in susceptibility to collagenases (decreased fibronectin levels) (reviewed in Winkler and Rath, 1999). Cervical dilatation during parturition is relatively rapid and involves increases in collagenase activity, elastase levels and enhanced invasion of the stroma by neutrophils. Neutrophil chemotaxis, activation and degranulation (release of proteases) are presumably under the influence of interleukin-8 (IL-8) from activated fibroblasts and macrophages. The exposure of the cervix to vaginal bacterial flora may activate these cells resulting in increased levels of inflammatory cytokines (tumour necrosis factor (TNF),

interleukin-1 (IL-1) and IL-8). These cytokines could then stimulate prostaglandin production which is responsible for the dilatation of cervical blood vessels and further invasion of leukocytes into the cervical tissue. Prostaglandins, especially prostaglandin E, are clinically used for cervical ripening (Calder and Embrey, 1973). Although oxytocin alone is adequate in stimulating contractions, oxytocin and prostaglandin E_2 in combination elicit a more efficient delivery because of cervical ripening (Huzar and Walsh, 1991).

b. Fetal Membrane Rupture

The fetal amnion and chorion are collectively referred to as the fetal membranes. During pregnancy, the fetal membranes envelop the fetus and bathe it in amniotic fluid and the chorion is firmly anchored to the maternal decidua. It is the structural responsibility of the fetal membranes to (1) possess enough strength and elasticity to withstand stretching due to fetal growth and movements, (2) withstand the additional strain of gravity conferred by an upright posture, and (3) retain their ability to rupture at term. A combination of mechanical distortion, enzymatic up-regulation and remodelling of the extracellular matrix are responsible for membrane rupture at term.

According to Bendon (1996), as uterine pressure increases, the uterus expands and the membranes stretch to accommodate this expansion with a resultant increase in membrane tension. When tension exceeds mechanical strength, the membranes tear. Membrane rupture usually occurs over the cervical os and may be related to cervical ripening. As the cervix dilates, the overlying avascular chorion membrane loses its blood supply from the maternal decidua and becomes deformed. This deformation may increase the tension of the fetal membranes beyond the limits of mechanical strength. Mechanical strength is dependant on

the strength of attachment of the adjacent membranes to the decidua. Alternatively, membrane rupture due to dilatation of the cervix may be mediated through exposure of the fetal membranes to vaginal bacterial flora (ascending infection) which causes inflammation and stimulates endogenous proteases. Stretch also weakens (thins) the membranes since the membranes are viscoelastic (i.e. cannot return to their original configuration after being stretched) (Lavery and Miller, 1977) and increases collagenase activity and IL-8 levels (Maradny *et al.*, 1996). Amniotic fluid from term pregnancies is an additional source of induced fibroblast collagenase production *in vitro* (Vadillo-Ortega *et al.*, 1991). Extracellullar matrix synthesis and turnover is continuous throughout pregnancy so that remodelling is due to mechanical disruption or an up-regulation of catabolic enzymes. Collagens are the major extracellular matrix components of fetal membranes and may be important for the attachment of chorion to decidua, dermatan sulfate helps to organize collagen, fibrillin-based microfibrils confer elasticity and glycosylated fibronectin (glycoprotein) in the chorionic cytotrophoblasts is important for matrix stabilization since it has multiple binding domains for cells and extracellular matrix components. Perturbation of the fibronectin-extracellular matrix links causes an increased production of collagenases. Perhaps the mechanical distension associated with fetal growth and movement reaches a point when cell-matrix contacts are lost, leading to enzyme induction, apoptosis, extracellular matrix degradation, membrane rupture and choriodecidual separation (Bryant-Greenwood, 1998).

c. Myometrial Contractility

The process of myometrial contraction and relaxation is mediated through the interaction of the major smooth muscle proteins, actin and myosin (reviewed in Word, 1995). More specifically, an increase in intracellular free calcium concentrations ($[Ca^{2+}]_i$) leads to the formation of a Ca^{2+}/calmodulin complex which can then bind myosin light chain kinase (MLCK). MLCK catalyzes the phosphorylation of a serine residue of the 20 kDa light chain of myosin. Phosphorylation results in conformational changes which lead to actin activation of myosin adenosine triphosphatase (ATPase) activity and shortening of the muscle. Relaxation occurs through decreases in $[Ca^{2+}]_i$ or phosphorylation of MLCK. Low calcium concentrations result in the de-phosphorylation of myosin by myosin phosphatase. The inactivation of MLCK so that it is unresponsive to activation by Ca^{2+}/calmodulin can be elicited by the phosphorylation of MLCK by cyclic adenosine monophosphate (cAMP)-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. Similarly, activation of adenylyl cyclase increases intracellular cAMP which decreases $[Ca^{2+}]_i$ and activates cAMP-dependent protein kinase A (PKA). The activity of the uterus is extensively regulated by the hormonal milieu and the mediators which influence myometrial contractility exert their effects through these signalling pathways either directly or via receptors (reviewed in Fuchs, 1995).

Electrical and metabolic coupling between myometrial cells is established through the formation of gap junctions which enables the uterus to contract in a synchronous fashion. The major gap junction protein is connexin-43 and six connexin molecules can interact to form a channel with a central pore through which small molecules can pass with little resistance. Thus, gap junctions are formed when connexins from adjacent cells interact.

2. REGULATION OF MYOMETRIAL CONTRACTILITY

a. The Role of Sex Steroids

Progesterone (21-carbon) and estrogens (18-carbon) are cholesterol-derived steroid hormones. There are three endogenous estrogens, namely estrone, 17β-estradiol and estriol which can exist in conjugated (biologically inactive) or unconjugated form (biologically active). The cascade summarizing the biosynthesis of progesterone and estrogens is depicted in Figure I-1. In non-pregnant ovaries, estrogen is produced in the granulosa cells under follicle-stimulating hormone (FSH) stimulation from androgen precursors provided by the theca cells. Following the luteinizing hormone (LH) surge and ovulation, the granulosa cells become the luteal cells of the corpus luteum and begin to secrete both estrogen and progesterone in response to human chorionic gonadotrophin (hCG). The corpus luteum either maintains its steroidogenic function throughout pregnancy in some species (cow, rabbit, rat and mouse), or functions only until the placenta is able to take over (sheep, non-human primates and humans) (Csapo, 1956). Neither progesterone nor estrogens can directly stimulate contractions of the uterus, but rather affect contractility through direct effects on transcription, protein synthesis, phospholipid membrane fluidity, ion balance, signal transduction and the synthesis of uterotonic agents.

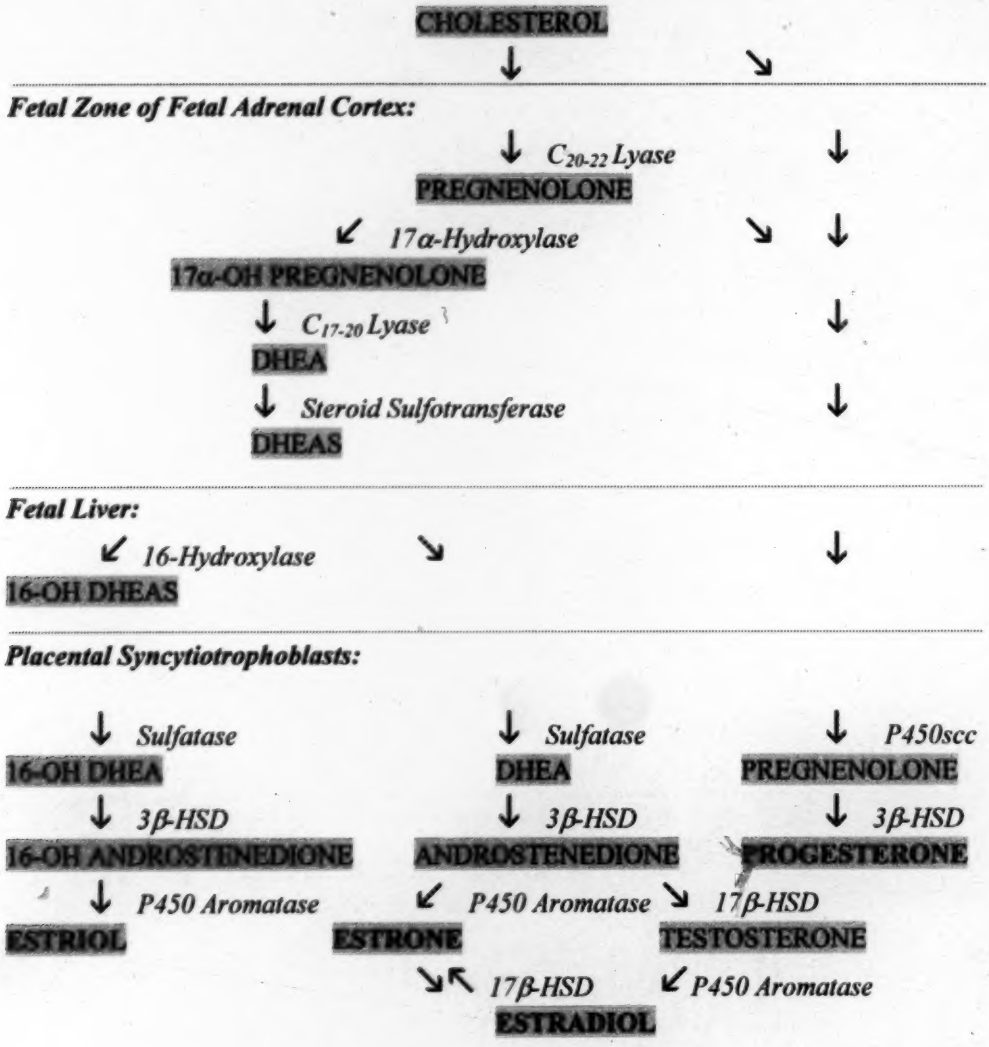

Figure I-1. The estrogen and progesterone biosynthetic cascade in humans. (DHEA = Dehydroepiandrosterone, DHEAS = Dehydroepiandrosterone Sulfate, 16-OH DHEA = 16-hydroxy-dehydroepiandrosterone, 16-OH androstenedione = 16-hydroxy-androstenedione, P450scc = P450 cholesterol side chain cleavage complex, 3β-HSD = 3β-Hydroxysteroid Dehydrogenase, 17β-HSD = 17β-Hydroxysteroid Dehydrogenase).

Progesterone Block

The progesterone block theory proposed by Csapo (1956) was based on observations that a dampening of spontaneous uterine contractility during pregnancy and the unresponsiveness of the myometrium to stimulation by oxytocin occurred during periods of progesterone dominance. Thus, progesterone is associated with uterine quiescence. In addition to its genomic actions, progesterone exerts its effects by altering the distribution of sodium and potassium ions across the myometrial cell membrane to disrupt ion balance and prevent efficient action potential propagation (Csapo, 1956). Similarly, progesterone enhances Ca^{2+} binding to the sarcoplasmic reticulum *in vitro* (Carsten, 1974). In addition, progesterone inhibits the expression of gap junctions (Puri and Garfield, 1982). However, the actin and myosin contractile apparatus was not affected by estrogen or progesterone dominance. Thus, a hallmark of this theory was the ease of reversal of the progesterone block. As a prerequisite for the termination of pregnancy, progesterone simply had to be withdrawn to restore efficient myometrial contractility (Csapo, 1956). Conversely, exogenous progesterone supplementation should suppress myometrial activity. A systemic progesterone withdrawal has been shown in rabbits, rodents and sheep (Csapo, 1956; Murr *et al.*, 1974; Thorburn and Challis, 1979).

However, the withdrawal of progesterone is not clearly substantiated in humans. Serum and plasma levels of progesterone either do not change or actually increase at term (Tulchinsky *et al.*, 1972; Challis *et al.*, 1974; Cousins *et al.*, 1977; Boroditsky *et al.*, 1978; Willcox *et al.*, 1985). Furthermore, treatment with exogenous progesterone failed to prevent imminent abortion or preterm labour (Csapo, 1956), although there was a decrease in the

frequency of preterm birth observed in women treated with 17α-hydroxyprogesterone caproate (reviewed in Myatt and Moore, 1994).

The Estrogen to Progesterone Ratio

Although there is a discrepancy between humans and other mammals with respect to the withdrawal of progesterone at birth, elevated estrogen levels appear to be a common link. The loss of progesterone dominance and emergence of estrogen dominance at birth is reflected by an increase in the estrogen to progesterone ratio (E:P ratio). Whereas progesterone is essential in the maintenance of uterine quiescence, estrogens are important in uterine activation or the preparation of the uterus for contraction. Estrogen promotes uterine contractile activity by increasing the synthesis of (1) actin and myosin (Csapo, 1969), (2) gap junctions (Puri and Garfield, 1982), (3) prostaglandin and oxytocin receptors (Alexandrova and Soloff, 1980), (4) the enzymes responsible for the production of prostaglandins (Myatt *et al.*, 1994; Wu *et al.* 1997), and (5) oxytocin (Chibbar *et al.*, 1995). Estrogens can also modulate membrane potential (Liggins *et al.*, 1977), regulate progesterone production in intrauterine tissues *in vitro* (Mitchell *et al.*, 1982; Wunsch *et al.*, 1986; Castracane and Goldzieher, 1986), and influence contractile frequency in non-labouring myometrium at term (Fu *et al.*, 1996).

Systemic Evidence

Evidence of a systemic increase in the E:P ratio has been well documented in animals but there is conflicting evidence of this increase in E:P ratio in humans. Unconjugated estrogen (estrone, estradiol and estriol) and progesterone levels in plasma gradually increased

towards term and the E:P ratio remained constant (Tulchinsky *et al.*, 1972). Several additional studies have shown no change in human term plasma and serum (Cousins *et al.*, 1977; Willcox *et al.*, 1985; Moutsatsou and Oakey, 1986). In contrast, Turnbull *et al.* (1974) showed an increase in the plasma estradiol to progesterone (E_2:P) ratio preceding labour at term and Boroditsky *et al.* (1978) reported an increase in serum estriol to progesterone (E_3:P) ratio. Studies measuring maternal salivary estriol as indices of free, unconjugated serum levels of estriol (Vining *et al.*, 1983) have also reported a surge in estriol levels and E_3:P ratios beginning about three weeks before delivery at term (Moran *et al.*, 1992; McGregor *et al.*, 1995). A novel method of achieving an increase in the E:P ratio was reported by Wilson *et al.* (1991). They have shown that there are nocturnal progesterone surges followed by estradiol surges in the pregnant baboon. Furthermore, two weeks prior to birth, the nocturnal estradiol surges shift forward to precede the progesterone surges thereby creating temporary windows of elevated E:P ratio which coincide with increased uterine activity. Whether a similar mechanism exists in humans requires further clarification.

Paracrine Evidence

Failure to find a consistent trend at the systemic level led to the idea that the local tissue concentrations were important. Khan-Dawood and Dawood (1984) showed that peripheral steroid concentrations from term, non-labouring women differed from tissue concentrations. Fetal membranes and decidua synthesize estrogens *in vitro* and this activity increases around the time of parturition (Mitchell *et al.*, 1984). Studies by Romero *et al.* (1988) have provided evidence for an increase in the E:P ratio in human amniotic fluid at term. Mitchell and Wong (1993) examined the levels of 17β,20α-hydroxysteroid

dehydrogenase (17β, 20α-HSD) in human chorion and chorion *in vitro*. This reversible enzyme acts on estrone and progesterone to reduce them to estradiol and 20α-dihydroprogesterone (20α-DHP), respectively. During pregnancy, the oxidative activity predominates with the preferred production of estrone and progesterone. As labour approaches, there is an increase in the reductive activity to favour increases in estradiol (more biologically active than estrone) and 20α-DHP (inactive metabolite of progesterone). This shift in activity could potentially lead to an increase in E:P ratio of chorion and amnion. However, it is unknown whether this is a cause or result of increases in uterine activity.

Estrogen and progesterone exert their genomic effects through the formation of ligand-receptor complexes which translocate to the nucleus and bind to nuclear sites to affect transcription. Consequently, a shift in the E:P ratio may also occur at receptor levels through an increase in the estrogen receptor (ER) to progesterone receptor (PR) ratio. In the lower myometrial segment, PR decreased in labour tissues (Giannopoulos and Tulchinsky, 1979). In choriodecidua, Chibbar *et al.* (1995) reported that ER mRNA increased at labour and PR mRNA remained unchanged. In contrast, How *et al.* (1995) have shown that myometrial ER does not change whereas term and preterm labouring myometrium had lower PR levels than tissues not in labour.

However, both ER and PR exist in several isoforms and the relative contributions of these isoforms have not been examined in detail. There are two receptors for estrogens, ERα and ERβ. At the amino acid level, they share 95% homology in the DNA-binding domain and 55% in the ligand-binding domain. Ligand bound ER forms homodimers or heterodimers (Cowley *et al.*, 1997) before binding directly to estrogen response elements (ERE) or to AP1 sites in conjunction with transcription factors. Using estradiol as the ligand, binding of ERα

or ERβ to ERE or binding of ERα to AP1 resulted in transcriptional activation whereas binding of ERβ to AP1 lead to the inhibition of transcription *in vitro* (Paech *et al.*, 1997). Thus, the relative proportions of each ER subtype could translate into significantly different activities. There are three PR isoforms (PRA, PRB, PRC) which differ in their interactions with other proteins (Kraus *et al.*, 1993). PR interacts with progesterone response elements (PRE) and studies *in vitro* have revealed that PRB and PRE interaction elicits more transcription than PRA and PRE.

Fetal Contribution

Extensive work has been done in sheep, non-human primates and humans with respect to the fetal contribution to labour through maturation and activation of the fetal hypothalamo-pituitary-adrenal (HPA) axis and the production of cortisol. This research has uncovered a complex pathway in which the fetus can initiate a cascade of events that ultimately leads to an increase in the E:P ratio. Elegant studies conducted by Albrecht, Pepe and colleagues (reviewed in Pepe and Albrecht, 1995; Albrecht and Pepe, 1999) have revealed a complex interaction between the human fetal adrenal gland, the placenta and the maternal circulation. In humans and primates, the fetal adrenal primarily produces DHEAS instead of cortisol and DHEAS serves as a substrate for the placental synthesis of estrogen (Albrecht and Pepe, 1990). Estrogen plays a crucial role by promoting the steroidogenic capacity of trophoblasts and modulating the enzymes responsible for the inter-conversion of cortisol and cortisone. During the second half of pregnancy, cortisol attenuates the maturation of the fetal HPA axis but around the time of parturition, a reduction in cortisol relieves the suppression of HPA axis maturation leading to the increased fetal adrenal

production of androgen precursors for estrogen biosynthesis. In humans, an additional unique pathway exists. DHEAS is converted to 16-hydroxy-DHEAS in the fetal liver which is then metabolized in the placenta to yield estriol.

b. The Role of Prostaglandins and Oxytocin

Following uterine activation, the uterus can be stimulated to contract through the action of uterotonic agents such as prostaglandins, oxytocin, endothelin-1 and other products of the arachidonic acid metabolism cascade. However, a thorough discussion of all of these mediators is beyond the scope of this thesis. Thus, the role of oxytocin will only be briefly addressed with the primary focus on prostaglandins.

Oxytocin and Oxytocin Receptor

Oxytocin is a potent stimulator of myometrial contractility. In 1906, an extract from the posterior pituitary was observed to stimulate contractions of the uterus (Dale, 1906) and the active ingredient of this extract was oxytocin. Oxytocin is a nonapeptide with an internal disulfide bond between cystine residues which forms a ring structure that is requisite for biological activity. Initial studies failed to find a pre-labour increase in plasma concentrations. However, in later studies, nocturnal surges in uterine contractions were correlated with serum oxytocin concentrations (Fuchs *et al.*, 1992). Subsequent studies revealed an intrauterine source of oxytocin which could signify a paracrine role for oxytocin. Oxytocin was higher in choriodecidua from women in term labour compared to tissues from women not in labour at term (Chibbar *et al.*, 1993). In term choriodecidual explants, oxytocin gene expression (mRNA and protein) are enhanced by estradiol and blocked by tamoxifen,

an estrogen receptor antagonist (Chibbar *et al.*, 1995). Oxytocin is used clinically to induce or augment labour and the pattern of labour observed is indistinguishable from spontaneous labour (Mitchell and Chibbar, 1995).

As parturition approaches, there are increases in decidual and myometrial oxytocin receptor protein (Fuchs *et al.*, 1982) and mRNA (Kimura, 1995) levels. The interaction of oxytocin with oxytocin receptor leads to the initiation of a signal transduction cascade that increases $[Ca^{2+}]_i$ and leads to myometrial contraction (Phaneuf *et al.*, 1993). The promoter of the oxytocin receptor gene possesses response elements suggestive of regulation by estrogens and pro-inflammatory cytokines. Myometrial cells in primary culture have responded to estrogen with increases in oxytocin receptor (Adachi and Oku, 1995).

Prostaglandins

Prostaglandins are 20-carbon unsaturated fatty acid chains that are important classical mediators of inflammation. The prostaglandins of primary interest in parturition are of the E and F series. They include, but are not limited to, prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and the $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM). Recent research substantiates a role for prostaglandins in the initiation and progression of parturition in humans at term. More specifically, a) there is an increase in prostaglandin levels at term, b) the inhibition of prostaglandin synthesis delays labour onset and prolongs labour, c) exogenous prostaglandins can induce labour, and d) prostaglandins cause uterine contractions *in vitro*.

Bioactivity Levels

In general, prostaglandin levels in maternal plasma, urine, amniotic fluid and intrauterine tissues increase late in gestation prior to labour onset and increase further with labour progression. Similarly, in women, plasma $PGF_{2\alpha}$ (Karim, 1968) and plasma PGFM levels (Mitchell *et al.*, 1978) increased with labour at term but were not elevated during late pregnancy. However, the half-life of prostaglandins in the circulation is less than one minute (Hamberg *et al.*, 1971) thereby suggesting that prostaglandins act in a paracrine fashion in the uterus. It was illustrated by Dray and Frydman (1976) that amniotic fluid PGE_2 and $PGF_{2\alpha}$ levels were higher during spontaneous labour than before labour onset. Furthermore, PGE_2 was present at higher levels than $PGF_{2\alpha}$ during pregnancy but the reverse was true as labour progressed (increased cervical dilatation). It was argued that enhanced prostaglandin production was a function of labour because the gradually opening cervix traumatized the surrounding tissues and allowed fetal membranes and decidua to contact the bacteria and cytokine-rich vaginal fluids which are potent stimulators of local prostaglandin production (MacDonald and Casey, 1993). Indeed, prostaglandin concentrations were elevated in samples obtained trans-vaginally when compared to samples obtained trans-abdominally from the same women (Romero *et al.*, 1994). Thus, in 1996(b), Romero *et al.* published studies using multiple trans-abdominal amniocentesis sampling to clearly depict pre-labour increases in amniotic fluid PGE_2 and $PGF_{2\alpha}$. Using dispersed cells from human amnion, chorion, decidua and placenta, Olson *et al.* (1983) reported that amnion and chorion PGE and PGF output was higher in women who laboured at term than those not in labour at term but this trend was not observed in decidua or placenta. These increases in bioactivity levels may

be associated with increased synthesis, decreased metabolism and increased expression of functional receptors.

Synthesis

The prostaglandin biosynthetic cascade is summarized in Figure I-2 and begins with arachidonic acid (5,8,11,14-eicosatetraenoic acid), a polyunsaturated fatty acid which is synthesized from linoleate, an essential fatty acid. Arachidonic acid is stored in esterifed form in the sn-2 position of glycerophospholipids. A rate limiting step in prostaglandin synthesis is the liberation of arachidonic acid from phospholipid membranes by phospholipases such as phospholipase A_2 (PLA_2) and C (PLC). There are five isoforms of PLA_2. Four are low molecular weight secretory forms (Types I-IV) which require millimolar concentrations of calcium for activity and one (Type V) is a high molecular weight non-secretory form in the cytosol ($cPLA_2$) which is active at micromolar concentrations of calcium (Rice, 1995).

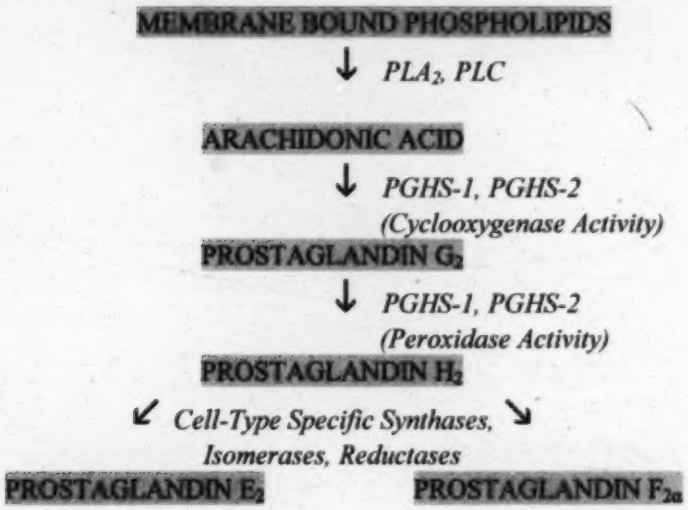

Figure I-2. The prostaglandin biosynthetic cascade. (PLA_2 = phospholipase A_2, PLC = phospholipase C, PGHS = prostaglandin endoperoxide H synthase).

Free arachidonic acid is then metabolized into prostaglandins and other eicosanoids. The prostaglandin pathway is mediated by prostaglandin endoperoxide H synthase (PGHS), synonymous with cyclooxygenase, in another rate-limiting step. Two heme-dependent functions are exhibited by PGHS including a cyclooxygenase catalytic activity which converts free arachidonic acid into prostaglandin G_2 and a peroxidase activity which catalyses the conversion of prostaglandin G_2 into prostaglandin H_2. Prostaglandin H_2 is then transformed into biologically active PGE_2 and $PGF_{2\alpha}$ by PGE isomerase or PGF reductase, respectively, in a tissue specific manner (Williams and DuBois, 1996). Existing as a membrane bound enzyme (Smith and DeWitt, 1995), PGHS also irreversibly self-inactivates following a finite number of catalytic reactions independent of substrate or product concentrations (Wu *et al.*, 1999). There are two isoforms of PGHS which share 60%

homology at the amino acid level and are products of different genes (Smith and DeWitt, 1995). The two isoforms also possess conserved catalytic amino acids and similar kinetic properties (Smith and DeWitt, 1995). Moreover, PGHS isoforms are highly conserved (~90%) between species (Loll and Garavito, 1994). Conventionally, PGHS-1 is thought to be constitutively expressed in most tissues (Masferrer *et al.*, 1994) where it plays a role in the regulation of vascular homeostasis, cytoprotection of the gastric mucosa, and renal water and sodium resorption. It has also been suggested that PGHS-1 expression is developmentally regulated (Smith and DeWitt, 1995). In contrast, PGHS-2 expression is induced by agonists including endotoxin, pro-inflammatory cytokines and mitogens (Herschmann, 1996) and accordingly, the PGHS-2 promoter contains transcription factor consensus sequences such as nuclear factor of interleukin-6 (NF-IL-6), cAMP and nuclear factor κB (NF-κB) (Williams and Dubois, 1996). Finally, PGHS-1 is located on the lumen side of the endoplasmic reticulum membrane and PGHS-2 is located in the endoplasmic reticulum and nuclear envelope (Smith and DeWitt, 1995).

Recent evidence suggests a role for both PLA_2 and PGHS in the regulation of parturition. Secretory Type II PLA_2 mRNA has been localized to human term gestational tissues including amnion, choriodecidua, and especially the placenta where it was most abundant (Aitken *et al.*, 1996; Freed *et al.*, 1997). Cytosolic PLA_2 mRNA is expressed principally in amnion although it was present in choriodecidua and placenta (Freed *et al.*, 1997). However, there is no change in secretory or cytosolic PLA_2 activity or Type II PLA_2 protein in amnion, choriodecidua or placenta before, during and after spontaneous labour at term (Munns *et al.*, 1999).

Teixeira *et al.* (1994) demonstrated that PGHS specific activity in the human amnion increases in late gestation before clinical signs of labour with further increases with the onset and progression of labour at term and preterm. The levels of PGHS-1 and -2 immunoreactive protein in the human amnion showed no change but it was postulated that this may be a consequence of the inability of the antibodies to distinguish between enzymatically inactive and active PGHS (Teixeira *et al.*, 1994). In contrast, at term Hirst *et al.* (1995) revealed that PGHS-2 mRNA levels, but not PGHS-1 mRNA levels, increase with the onset of spontaneous labour in the human amnion compared to amnion obtained at term by caesarean section with no clinical signs of labour. In addition, elevated PGHS-2 mRNA synthesis correlated positively with increased enzyme activity. Similar results were observed by Mijovic *et al.* (1997) in the chorion at term, but no change in mRNA levels was observed in human decidua at term (Hirst *et al.*, 1998). PGHS-1 and 2 mRNA were significantly elevated when comparing term to preterm non-labouring chorion and activity correlated with PGHS-2 (Mijovic *et al.*, 1998). Zuo *et al.* (1994) showed that human myometrium expressed PGHS-1 and -2 but Moore *et al.* (1999) detected no changes PGHS-1 and -2 mRNA in lower myometrial segments with gestational age or labour at term and preterm.

Metabolism

Biologically active PGs are converted into metabolites with reduced bioactivity primarily by a nicotinamide adenine dinucleotide (NAD+)-dependent 15-hydroxyprostaglandin dehydrogenase (PGDH). The formation of 15-keto and 13,14-dihydro metabolites occurs through the oxidation of the 15-hyroxyl group of the PGE and PGF series by PGDH (Challis *et al.*, 1997).

Mitchell *et al.* (1977) in amniotic fluid and Olson *et al.* (1983) in intrauterine tissues suggested that the increase in prostaglandin output with labour onset is a consequence of increased synthesis rather than decreased metabolism since the levels of $PGF_{2\alpha}$ (and its stable metabolite, PGFM) remained constant relative to each other regardless of the tissue examined or the cause of labour onset. Furthermore, Mitchell *et al.* (1993b) demonstrated that the metabolism of prostaglandins by PGDH increased around the time of parturition, was highest in chorion explants and more active for PGE_2 than $PGF_{2\alpha}$ and that transfer of radiolabelled PGE_2 was low, making it unlikely that amnion PGE_2 reaches myometrium. Intrauterine prostaglandin catabolic enzymes do not change or decrease during labour (Sangha *et al.*, 1994; Van Meir, 1996). Chorion has a lot of PGDH activity which may regulate the amount of prostaglandins in amniotic fluid from reaching the site of action in myometrium and ideal because interposed between amnion and decidua. In chorion, PGDH activity predominates (Nakla *et al.*, 1986) and PGDH activity is lost late in gestation (Challis *et al.*, 1997). Furthermore, basal PGDH activity is lower in human placenta or chorion obtained during labour at term than at elective caesarean section (Patel *et al.*, 1999). Studies using RU486 or trilostane also suggest that PGDH activity is increased by progesterone *in vitro* (Patel *et al.*, 1999).

Receptors

Prostaglandin $F_{2\alpha}$ (acting on FP receptors) and PGE_2 (acting on EP_1 receptors) cause myometrial contraction in humans by activating the phospholipase C signal transduction pathway and increasing intracellular calcium concentrations. Myometrial contractions also result from the activation of EP_3 receptors by PGE_2 to inhibit adenylate cyclase. Conversely,

PGE_2 acting on EP_2 receptors and the recently discovered EP_4 receptor cause relaxation by activating adenylate cyclase to increase cAMP levels and decrease cytosolic calcium levels (Fuchs, 1995; Nishigaki *et al.*, 1998).

With regard to receptor expression, Giannopoulos *et al.* (1985) have demonstrated that myometrial prostaglandin receptor levels do not change during late gestation in humans. In pregnancy, EP_3 and FP gene expression in human myometrium was reduced when compared to non-pregnant myometrium, thus helping to maintain uterine quiescence in humans (Matsumoto *et al.*, 1997).

Inhibition

Inhibition of prostaglandin synthesis delays labour onset, reduces contractions, and prolongs the labour process (Lewis and Schulman, 1973; Reiss *et al.*, 1976). Vane (1971) was the first to demonstrate that inhibition of prostaglandin synthesis was the mechanism of action for aspirin-like drugs. A retrospective study of pregnant women who had taken aspirin during the last two trimesters of pregnancy by Lewis and Schulman (1973) showed that these women had significantly longer gestations (approximately one extra week) and extended duration of labour when compared to controls. Early PGHS synthesis inhibitors did not differentiate between PGHS-1 and PGHS-2. Recently, Bhattacharyya *et al.* (1995) established valerylsalicylic acid as a selective inhibitor of PGHS-1. However, most of the current drug development is directed against PGHS-2 (Pairet and Engelhardt, 1996). Ideally, specifically blocking PGHS-2 may abolish the adverse side effects associated with inhibition of PGHS-1 and have potent anti-inflammatory effects. Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting prostaglandin synthesis.

Exogenous Prostaglandin Administration

The first successful induction of labour at term by prostaglandins ($PGF_{2\alpha}$) administered intravenously was reported by Karim *et al.* (1968). Both PGE_2 and $PGF_{2\alpha}$ are effective in cervical ripening and the initiation of myometrial contractions at any time during pregnancy thereby rendering them clinically useful in inducing abortions in early pregnancy or labour at term (Mitchell, 1981).

Prostaglandin Actions

Since their discovery, prostaglandins have been associated with reproduction. Kurzrok and Lieb (1930) first reported that human semen was able to both inhibit and stimulate human uterine contractions, *in vitro*, and subsequently, Von Euler (1935) and Goldblatt (1935) determined that factors within human seminal fluid could stimulate smooth muscle to contract. These factors were tentatively named prostaglandins by Von Euler (1935) by virtue of their proposed origin in the prostate gland of human males, a supposition later proven incorrect. The isolation, purification and structure of prostaglandins then ensued and was accomplished through the combined efforts of Bergstrom and Samuelsson (Bergstrom and Sjovall, 1957; Bergstrom and Samuelsson, 1968). Prostaglandins are potent myometrial contractile agents (uterotonins) which stimulate uterine contractility *in vitro*. Prostaglandin $F_{2\alpha}$ is a potent stimulator of uterine contractions and generates an increase in tension in a dose-dependent manner when added to pregnant human myometrium whereas PGE_2 caused an initial contraction followed by a period of relaxation. Interestingly, pregnant myometrium was more refractive to the contractile effects of PGE_2 and $PGF_{2\alpha}$, but this may be attributed to gestational age-related effects (Word *et al.*, 1992). Thus, $PGF_{2\alpha}$ seems to be more

important for enhancing uterine contractile activity while PGE_2 plays a role in fetal membrane rupture and cervical ripening (Olson *et al.*, 1995).

In addition to their contractile activities, Word *et al.* (1992) suggests that prostaglandins may increase the production of other uterotonins and increase the sensitivity of myometrium to uterotonins. Prostaglandins also increase the synthesis of gap junctions (Garfield *et al.*, 1980) and alter the hormonal milieu of pregnancy.

c. The Role of Cytokines

The Fetal Allograft

The concept of pregnancy is miraculous not only by virtue of the creation of life, but also because of its paradoxical existence. In 1953, Medawar was one of the first individuals to point out the fact that the genetically distinct fetal-placental unit possesses a complement of paternal and developmental antigens which are able to survive despite potential recognition and rejection by a competent maternal immune system. Several theories have evolved in attempts to explain this contradiction and focus on the placenta as the primary interface and physical barrier between the intimately juxtaposed mother and fetus. They include (1) sequestration of the fetus within a shell of trophoblasts which exhibit a diminished and restricted presentation of major histocompatibility complex (MHC) antigens which themselves may be necessary to prevent natural killer cell-mediated non-specific cell death, (2) camouflage of antigens by blocking antibodies which can recognize the antigen but do not elicit an immune response, (3) destruction of antibodies in transit across the placenta by receptors for the constant region of antibodies and (4) modifications of the

maternal immune system. Each of these theories are not sufficient in themselves to explain the survival of the fetal allograft but the combination of these theories is probably a reasonable estimate of the events *in vivo*. For a thorough review of the first three mechanisms, please refer to Hunt and Orr, 1992; Billington, 1992; Herrera-Gonzalez and Dresser, 1993; Wood, 1994; and Clark *et al.*, 1996. The fourth mechanism is an important focus of this thesis because there is considerable speculation that parturition at term may simply be a rejection of the fetus at the appropriate time (Lundin-Schiller and Mitchell, 1990). How this time is determined remains the subject of current research efforts.

Modification of the Maternal Immune System

Relevant Uterine Immune Cell Populations

Many studies investigating the haemopoietic immune cell populations in the uterus have been conducted with respect to implantation and the establishment of pregnancy. There has been very little research done on the changes in cell populations in the context of parturition and labour onset. Nevertheless, macrophages have been detected in decidua and myometrium at sites distant from the placenta and they tend to increase in density during pregnancy (Hunt *et al.*, 1985). Natural killer (NK) cells are located in the metrial glands, decidua and labyrinthine region of the placenta (Croy *et al.*, 1997). There is some evidence that an abnormal accumulation of NK cells in the decidua is associated with recurrent spontaneous abortions (Yamamoto *et al.*, 1999). In addition, there is an increase in NK cell activity before human parturition (Szekeres-Bartho *et al.*, 1986). T-helper lymphocytes (TH cells) are present, but only make up a small proportion of the uterine immune cells.

Cytokines are signalling proteins that are released from activated cells and act locally as regulators of cell function. They represent an important communication network both within the immune system and between the immune system and other physiological systems. The haemopoietic immune cells are important sources of cytokines, but they are not the only source. Cytokines may also be produced by uterine epithelium and trophoblasts (Pollard *et al*, 1987; Branch *et al.*, 1987; Robertson *et al.*, 1992; Hunt, 1994) which are situated in a position to recognize and respond to foreign fetal antigens.

Humoral Immunity Bias in Pregnancy

Although women are not markedly immunocompromised, they do exhibit a tendency to favour humoral immune responses (antibody-mediated) over cell-mediated immune (CMI) responses (mediated by cytotoxic T lymphocytes (CTL) and NK cells) during pregnancy. Both branches of immunity can be regulated by TH cells via the release of specific cytokines. In women, there is an increased susceptibility to diseases that are normally resolved by CMI (i.e. viral and intracellular pathogens) and a decreased susceptibility to diseases which elicit humoral responses. Furthermore, pathophysiological conditions in which an aberrant CMI response dominates (e.g. CTL in joints of patients with rheumatoid arthritis) experience remission (Da Silva and Spector, 1992) while inappropriate humoral dominance (e.g. excessive autoantibodies in systemic lupus erythematosus) results in the exacerbation of symptoms (Varner, 1991) during pregnancy.

The TH1 vs. TH2 Paradigm

The TH cell is central to both CMI and humoral immunity. TH cells differentiate into three functional subsets which are based upon their cytokine secretion profile. TH1 cells secrete interleukin-2 (IL-2), interferon-γ (IFN-γ), tumour necrosis factor-β (TNF-β) and TH2 cells produce interleukin-4 (IL-4), interleukin-6 (IL-6) and interleukin-10 (IL-10) while TH0 cells produce both cytokine subsets (Mosmann *et al.*, 1986; Fiorentino *et al.*, 1989). Interleukin-3 (IL-3), tumour necrosis factor-α (TNF-α), and granulocyte-monocyte colony stimulating factor (GM-CSF) are recent additions to the expanding subsets and are common to both the TH1 and TH2 subsets (Mosmann and Sad, 1996). There may also be a potential surface marker of TH2 cells called CD30 (Piccinni *et al.*, 1995). These subsets are mutually inhibitory because IFN-γ inhibits TH2 proliferation while IL-10 inhibits TH1 cytokine synthesis (Mosmann and Coffman, 1989). It is now recognized that other cells can produce these cytokine subsets so TH1 and TH2 have come to refer to the cytokine profiles themselves, not necessarily the cells which produce them.

It is the TH2 suppression of TH1 which is most crucial in allograft survival (Strom *et al.*, 1996). TH1 cytokines can activate macrophages, stimulate CTL and NK cells, and are involved in CMI and graft rejection. Excessive uterine NK cell activation could lead to trophoblast damage (Moreau *et al.*, 1998). TNF can stimulate apoptosis of human primary villous trophoblasts and this effect can be enhanced by IFN-γ (Garcia-Lloret *et al.*, 1996). Conversely, TH2 cytokines stimulate the development of B lymphocytes and the production of immunoglobulin E (IgE) and G (IgG) antibodies in humoral immunity. Furthermore, IL-4 plays a key role in determining the differentiation pathway of naive TH cells (Swain, 1993; Kopf *et al.*, 1993).

Several observations led to the linking of TH2 cytokine profiles with pregnancy success (Robertson *et al.*, 1994). Firstly, there was a shift in maternal immunity towards the humoral branch of the immune system during pregnancy (Varner, 1991; Da Silva and Spector, 1992). Secondly, there is a correlation between humoral immunity and the TH2 profile of cytokine production (above). Lastly, the beneficial cytokines associated with enhanced placental growth and fetal survival belonged to the TH2 subset whereas those cytokines associated with growth limitation and deleterious effects in gestation were members of the TH1 subset (Robertson *et al.*, 1994). In 1993, it was hypothesized by Wegmann and colleagues that the maternal immune system preferentially induces a TH2 response during successful pregnancy and thereby down-regulates production of harmful TH1 cytokines which lead to pregnancy loss (Wegmann *et al.*, 1993). In this context, it is speculated that parturition at term or preterm represents a shift in favour of a TH1 profile.

Pro-inflammatory vs. Anti-inflammatory

The presence and availability of immune cells in the uterus and their requisite role in the establishment and maintenance of normal pregnancy as well as the association of inflammatory mediators such as prostaglandins with parturition has led to the suggestion that parturition can be likened to an inflammatory response. Consequently, pregnancy would be characterized by a dominance of anti-inflammatory mediators at the fetal-maternal interface which would suppress or attenuate the activation of the immune cells. Pro-inflammatory cytokines could potentially be up-regulated at the time of parturition to initiate the inflammatory response. Inflammation involves immune cell recruitment and infiltration, activation of these cells leading to cytokine production, and cell interactions which may

modulate prostaglandin production leading to cell destruction and rejection of the fetus. Pro-inflammatory cytokines include TNF-α, IL-1α, IL-1β, IL-6, and IL-8 and can be released by most cells following injury, but activated macrophages are important sources (Dudley and Trautman, 1994). The repertoire of anti-inflammatory cytokines include IL-10, transforming growth factor-β (TGF-β), IL4 produced by T cells, B cells and macrophages. Interleukin-10 was also known as cytokine synthesis inhibitory factor because of its inhibition of the synthesis of pro-inflammatory cytokines. Although these profiles share a striking similarity to the TH1 and TH2 profiles, they are distinctly unique in their ability to elicit specific immune responses.

Evidence Supporting a Role for Cytokines

In normal human pregnancy, peripheral blood mononuclear cells (PBMC) exhibit a decrease in TH1 cytokines (IL-2 and IFN-γ) accompanied by an increase in TH2 and anti-inflammatory cytokine (IL-4 and IL-10) production following antigen stimulation (*Marzi et al.*, 1996). Only low levels of IL-10 mRNA were detected in PBMC throughout pregnancy (Tranchot-Diallo *et al.*, 1997). Supernatants from trophoblast-activated PBMC in normal pregnancies contained IL-10 but no TH1 type cytokines (Hill *et al.*, 1995). Interleukin-4 and IL-10 have been localized to trophoblasts and decidual macrophages (de Moraes-Pinto *et al.*, 1997). Soluble TNF receptors (TNFR p55 & p75) which would play an anti-inflammatory role increased between the second and third trimester in plasma (Kupferminc *et al.*, 1995).

With labour at term in humans, the role of these cytokine subsets is not as clear and distinct. There is a decrease in plasma and amniotic fluid TNF-β (Laham *et al.* 1997) at term. In human decidua obtained at term in labour or not in labour, messenger ribonucleic acid

(mRNA) was detected for IL-10 and IFN-γ, but the corresponding protein levels *in vitro* showed that IFN-γ decreased in labouring tissues. Furthermore concentrations of IFN-γ and IL-10 were detected in the amniotic fluid, but no change in these levels was observed. Thus, there is evidence that labour is not associated with increased levels of TH1 cytokines (Jones *et al.*, 1997). The authors also suggested that the decrease in IFN-γ is a consequence of labour rather than labour onset since similar results were obtained from spontaneous vaginal deliveries and induced vaginal deliveries. Using fetal membranes in primary culture, Trautman *et al.*, (1997) suggested that the decidua is the primary source of IL-10 at term. Amniotic fluid IL-10 is detectable but does not change with labour (Dudley *et al.*, 1997) and low levels of IL-10 were observed during placental perfusion (Denison *et al.*, 1998). In contrast, basal IL-10 levels in choriodecidual explants decreased with labour onset (Simpson *et al.*, 1998). Term and threatened preterm labour are associated with increased lymphocyte cytotoxicity in blood (Szekeres-Bartho *et al.*, 1986). When compared to non-labouring women, labour at term was associated with higher levels of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) in amniotic fluid (Romero *et al.*, 1992a, b, and c; Opsjon *et al.*, 1995; Gunn *et al.*, 1996), fetal membranes (Dudley *et al.*, 1996b; Gunn *et al.*, 1996), placental and decidual cell cultures (Steinborn *et al.*, 1996a and b, Steinborn *et al.*, 1998), choriodecidual explants (Laham *et al.*, 1996a; Laham *et al.*, 1996b) and decidua (Ammala *et al.*, 1997). However, the relevance of amniotic fluid cytokines on activities outside of the amniotic cavity is debatable since only a low percentage (2-17%) of IL-1α, IL-1β, TNF-α, and IL-6 is transferred across term, not in labour, fetal membranes *in vitro* (Kent *et al.*, 1994). Thus, during parturition at term, there appears to be stronger evidence favouring a role for pro-

inflammatory cytokines but the influence, if any, of the other cytokine profiles need to be further elucidated.

Controversies

The controversy regarding the TH1 and TH2 paradigm arises from the lack of evidence for a direct cause and effect relationship. Most of the studies have investigated the role of this paradigm in early pregnancy and there is very little information on its role, if any, during parturition. In addition, is there really any advantage to favouring the effects of one subset over the other? It may be argued that a bias towards an antibody-mediated immune response is better tolerated in pregnancy because the placenta acts as an immunoadsorbent (Wegmann, 1979) that can remove large amounts of anti-fetal antibodies without damage, and the increase in antibodies may confer a greater degree of passive immunity to the fetus (Wegmann, 1993). Similarly, the pro-inflammatory and anti-inflammatory scheme suffers from an inability to determine whether the increases are a cause of labour, or a consequence thereof (Cox *et al.*, 1993). Finally, immune responses form a continuum and the paradigms described above are over-simplifications which, although convenient, fail to do justice to the broad spectrum of responses possible (Dudley, 1995).

C. HUMAN PRETERM PARTURITION

Instances in which parturition occurs prior to thirty-seven weeks in humans are classified as preterm (World Health Organization, 1977). In this context, abortions may represent extreme cases of preterm birth. Preterm birth occurs in 5-11% of all deliveries

(Ventura *et al.*, 1997; Health Canada, 1996) and is the primary cause of perinatal morbidity and mortality (Health Canada, 1996; MacDorman and Atkinson, 1998). In 1995, 64% of all infant deaths in the United States occurred in infants born preterm (MacDorman and Atkinson, 1998). As the gestational age at birth increases, the health and survival of the preterm neonate also increases which substantiates the need for the development of various tocolytic drugs to stop preterm uterine contractions and prolong gestation. Even a short delay in the onset of labour may be adequate to transfer the mother and fetus to facilities specialized in the care of preterm infants. Regardless of these efforts, the risk of preterm birth has not changed significantly in the past four decades (Ventura *et al.*, 1997). Furthermore, the Canadian Consensus Workshop Group (1995) studied the efficacy and side effects of several tocolytic drugs including ritodrine (beta-adrenoreceptor agonist), magnesium sulphate (calcium ion antagonist), indomethacin (inhibitor of prostaglandin synthesis), atosiban (oxytocin receptor antagonist), nifedipine (calcium channel blocker), glyceryl trinitrate (nitric oxide donor) and antibiotics. They concluded that although only ritodrine and indomethacin were effective in prolonging gestation, neither was effective in improving neonatal outcomes.

Advances in neonatal care have improved the survival rate of preterm infants. However, these infants are more susceptible to severe respiratory distress syndrome, intraventricular haemorrhage, necrotizing enterocolitis, sepsis, and growth restriction (Dusick, 1997) as well as long-term problems such as neurologic deficits, chronic lung disease, deafness, blindness, educational difficulties, and family disruption (Novy *et al.*, 1995; Corbet *et al.*, 1991; Bennett and Scott, 1997; Vohr and Msall, 1997; Leonard and Piecuch, 1997). This translates into significant emotional, financial and societal problems.

In 1991, Canada spent an estimated $11.1 million per day caring for preterm infants (Health Canada, 1996).

Factors predisposing a pregnant woman to preterm birth include poor socioeconomic status, maternal age, low body mass index, health, life style and work habits, genetics, multiple pregnancies, prior history of abortions, excessive alcohol consumption, maternal and intrauterine infection, and the level of prenatal care (reviewed in Stubblefield, 1993). However, the majority of aetiologies are idiopathic (Health Canada, 1996).

1. THE ROLE OF ALCOHOL

An association between alcohol and adverse pregnancy outcomes has been well documented. When discussing the effects of alcohol, it is important to distinguish between the different patterns of alcohol consumption, namely chronic or acute. Chronic alcoholism in pregnancy has received a great deal of attention due to the large interest in fetal alcohol syndrome. With respect to parturition, it was observed that gestational lengths in animal models of chronic alcohol consumption were increased (Chen and Smith, 1979; Fuchs, 1966; Hahn et al, 1984; Leichter and Lee, 1979: Maldaner et al, 1994) and infusions of ethanol to pregnant women decreased spontaneous uterine contractions (Fuchs *et al.*, 1967; Zlatnik and Fuchs, 1972). This effect was later attributed to the inhibition of maternal pituitary oxytocin release (Fuchs, 1966). However, clinical application of this observation has not come into fruition (Zervoudakis *et al.*, 1980).

In contrast, there is little data available on acute doses of alcohol and pregnancy even though acute alcohol consumption may be the more prevalent pattern. Ebrahim *et al.* (1999),

have documented an actual increase from 1991 to 1995 in the prevalence of binge-drinking during pregnancy in the United Sates. Very few studies have examined alcohol consumption as a risk factor for preterm birth and those that did showed discrepancies in their results and often did not differentiate between chronic and acute exposure during pregnancy. There are conflicting reports that alcohol consumption has no association with preterm birth (Shiono *et al.*, 1986; Peacock *et al.*, 1995; Windham *et al.*, 1995; Mercer *et al.*, 1996), decreases the risk of preterm birth (Meis *et al.*, 1998), or increases the risk (Olegard *et al.*, 1979; Hingson *et al.*, 1982a and b; Berkowitz *et al.*, 1982; Borges *et al.*, 1993, Lazzaroni *et al.*, 1993). Understandably, the rates of acute alcohol exposure during pregnancy are probably underestimated due to an inherent reluctance of women to admit to alcohol use during pregnancy.

2. THE ROLE OF SEX STEROIDS

Serial measurements of steroids were made in high risk pregnancies that eventually delivered preterm and these experiments showed no change in mean plasma estradiol or progesterone concentrations (Block *et al.*, 1984). However, there is an increase in the maternal salivary E_3:P ratio approximately three weeks before preterm labour onset (McGregor *et al.*, 1995; Goodwin, 1999). In addition, during preterm labour there is no change in progesterone but amniotic fluid and plasma estradiol levels are higher than levels during term labour (Mazor *et al.*, 1994). Epostane leads to abortion in the second trimester in humans (Selinger *et al.*, 1987).

3. THE ROLE OF PROSTAGLANDINS

Teixeira *et al.* (1994) demonstrated that PGHS specific activity in the human amnion is elevated during preterm labour. In contrast to the pattern observed at term, both PGHS-1 and -2 mRNA were enhanced in chorion obtained from women in preterm labour when compared to preterm not in labour groups (Mijovic *et al.*, 1998). Furthermore, intra- or extra-amniotic $PGF_{2\alpha}$ and PGE_2 are effective abortifacients in early or middle pregnancy (Kent, 1999).

4. THE ROLE OF CYTOKINES

Disruption of the immunological adaptations necessary for the survival of the fetal allograft may be causative factors in the pathophysiology of abortions and preterm birth. However, it is questionable whether findings from either abortions or preterm birth can be extended to each other. The majority of the evidence supporting the involvement of cytokines in relation to preterm birth is derived from cases of intrauterine infection. Maternal and intrauterine infection are major causes of preterm labour and birth (reviewed in Gibbs *et al.*, 1992; Romero *et al.*, 1993; Dudley and Trautman, 1994). Systemic maternal infections including pyelonephritis, pneumonia, malaria and typhoid fever are associated with the onset of labour, and localized intrauterine infection is associated with preterm labour and delivery. What appears to be important is not the direct toxicity of the stimulus or insult, but rather the host response to the stimulus and whether it is appropriate (Dudley and Trautman, 1994). In attempting to destroy the invading organism, a cascade may be initiated that inadvertently

results in delivery of the fetus. Alternatively, the induction of preterm labour may be the difference between survival and death (Mazor *et al.*, 1998).

Abortions

In human recurrent spontaneous abortions, IFN-γ and TNF are detected in trophoblast-activated PBMC (Hill *et al.*, 1995). Furthermore, a dominant TH1 and diminished TH2 profile in PBMC was associated with pathological pregnancies (Marzi *et al.*, 1996). However, only 50% of abortions have an identifiable cause and immunization with paternal leukocytes has only been partially successful (Dudley, 1995).

Preterm Labour

Women with malaria delivered low birth weight infants and had higher than normal IFN-γ and TNF-α levels and lower than normal IL-10 in their placentas (Fried *et al.*, 1998). In amniotic fluid, IL-10 was detectable but did not change with preterm labour, even in presence of infection (Dudley, 1997). Infection associated preterm labour was characterized by elevated amniotic fluid levels of IL-1 (Romero *et al.*, 1989b), IL-4 (Dudley *et al.*, 1996c), TNF (Romero *et al.*, 1989a), IL-6 (Romero *et al.*, 1990), IL-8 (Romero *et al.*, 1991a). Preterm labour without infection had elevated IL-1β, IL-6 and TNF in placental/decidual cell cultures (Steinborn *et al.*, 1996) and amniotic fluid (Arntzen *et al.*, 1998). Furthermore, amniotic fluid soluble TNF receptor was lower in preterm birth without infection. Placental macrophages also produced higher TNF-α levels during preterm labour (Steinborn *et al.*, 1998). In the second trimester, elevated amniotic fluid IL-6 may be valuable predictors of preterm labour (Ghidini *et al.*, 1997); Wenstrom *et al.*, 1998).

D. ANIMAL MODELS OF PRETERM LABOUR

As a consequence of moral and ethical considerations and the relative difficulty in obtaining tissues from pregnant women, there is a paucity of knowledge about the events precipitating preterm birth in humans. This is evident from the brevity of the preceding section of this thesis. It therefore becomes necessary to explore the use of animals in further elucidating the mechanisms responsible for the phenomenon of parturition. While it is acknowledged that some mechanisms are unique to humans and vice versa, there are also some points of convergence which may prove applicable to humans.

1. MANIPULATION OF SEX STEROIDS

The manipulation of sex steroids are extensively used to induce preterm birth in animal models. This induction itself is indicative of the importance of steroids in the regulation of parturition.

Induced Progesterone Withdrawal

The maternal administration of RU486 (Mifepristone), a progesterone receptor antagonist, can induce preterm labour in mice (Dudley *et al.*, 1996a) and rhesus monkeys (Haluska and Novy, 1993). Although the administration of RU486 to rhesus monkeys increased uterine activity, it failed to initiate the normal cascade of events observed during normal spontaneous birth at term (Haluska et al., 1987; Haluska and Novy, 1993). In

humans, RU486 in conjunction with prostaglandins is often used clinically as an abortifacient.

Inhibitors of 3β–HSD such as epostane or trilostane can induce preterm uterine activity and birth in rhesus monkeys (Haluska *et al.*, 1997), and this effect in rhesus monkeys was blocked by progesterone implants (Haluska *et al.*, 1997). However, epostane also inhibits cortisol and androstenedione production (which is a precursor for estrogen).

Induced Estrogen Dominance

In sheep, the administration of exogenous estrogen induces labour (Hindson *et al.*, 1967) but the labour was abnormal because birth was often delayed due to a failure of the cervix to dilate. In contrast, subcutaneous estrogen was ineffective in rhesus monkeys (Novy and Walsh, 1983) and baboons (Albrecht *et al.*, 1989), which may be a consequence of the mode of treatment delivery. Peripheral estrogen infusion was able to induce myometrial activity in rhesus monkeys, but this was also not followed by preterm delivery (Nathanielsz *et al.*, 1998).

On the other hand, enhanced endogenous estrogen production induced by glucocorticoid (cortisol, dexamethasone, betamethasone, ACTH) infusion to the fetus resulted in the preterm birth of fetal sheep (Liggins, 1968). Similarly, the maternal infusion of androstenedione to rhesus monkeys increases placental estrogen production and initiates contractions and birth (Mecenas *et al.*, 1996). An increase in maternal plasma estradiol preceded the plasma oxytocin increase (Giussani *et al.*, 1996) in this model, and this could be abolished by co-treatment with aromatase inhibitors such as 4-hydroxyandrostenedione (Nathanielsz *et al.*, 1998). This also prevented the androstenedione-induced changes leading

to birth. This discrepancy between direct and indirect methods of inducing estrogen dominance suggested that estrogen synthesis must be stimulated at its physiological site of production to allow it to have its systemic endocrine (contractions) and local paracrine actions (membranes, cervix *etc.*) (Nathanielsz *et al.*, 1998). Furthermore, the importance of distinctions between endocrine and paracrine levels of regulation was underlined.

2. MANIPULATION OF PROSTAGLANDINS

Induced Prostaglandin Increase

Again, further evidence of a role for prostaglandins in preterm labour is derived from its ability to induce preterm labour. Infusion of PGE_2 to fetal sheep results in significantly shorter gestational lengths (Young *et al.*, 1996) and is accompanied by increases in plasma ACTH and cortisol (Hollingworth *et al.*, 1995). Prostaglandin $F_{2\alpha}$ can also induce labour in rhesus monkeys (Challis, 1974).

3. MANIPULATION OF CYTOKINES

Induction by Bacteria or Bacterial Products

Intravenous administration of *Salmonella* endotoxin leads to the preterm delivery of fetal lambs (Schlafer *et al.*, 1994) following fetal hypoxemia and activation of the fetal HPA axis. Intra-amniotic inoculation of chronically instrumented rhesus monkeys with group B *streptococci* forms the basis of a non-human primate model of preterm birth and provides evidence for a causal relationship between intra-amniotic infection and preterm labour

(Gravett *et al.*, 1994). This model also had increased production of IL-1 receptor antagonist in amniotic fluid (Witkin *et al.*, 1994). Intrauterine infection with *Escherichia coli* leads to preterm labour in mice (Hirsch *et al.*, 1995). In association with preterm birth in this model, there was an induction of the expression of mRNA for TNF-α, IL-1α and β, PGHS-2 as well as protein for IL-1α and β, IL6 and TNF-α in the uterus (Hirsch *et al.*, 1999). Intracervical inoculation of *E. coli* is also an effective method of inducing preterm birth in mice and is followed by uterine PGHS-2 expression and pro-inflammatory cytokine (IL-1β, TNF-α, IL-6) expression (Reznikov *et al.*, 1999). Mice deficient in IL-1β could undergo *E. coli*-induced preterm birth despite reduced uterine IL-1β, TNF-α, and IL-6 expression relative to wild-type mice (Reznikov *et al.*, 1999). Intrauterine infection with *E. coli* in rabbits leads to increased NF-κB which induces production of pro-inflammatory cytokines (Leslie *et al.*, 1999).

Several murine models utilize a bacterial endotoxin, lipopolysaccharide (LPS), to trigger fetal death and abortion during early gestation (Gendron *et al.*, 1990; Silver *et al.*, 1994; Silver *et al.*, 1995; Silver *et al.*, 1997) and this death can be ameliorated with anti-TNF antibodies (Silver *et al.*, 1994) or anti-TNF drugs (Gendron *et al.*, 1990). Resorptions are associated with elevations in amniotic fluid TNF-α and NK cell infiltration of placentas (Gendron *et al.*, 1990). Decidual explants from LPS-exposed mice also produce more PGHS-2, PGE_2 and $PGF_{2\alpha}$ than controls (Silver *et al.*, 1995).

The administration of LPS also induces preterm birth in mice (Fidel *et al.*, 1994; Kaga *et al.*, 1996) and treatment was associated with increased amniotic fluid IL-1α, IL-6 but with no change in TNF-α (Fidel *et al.*, 1994). Treatment with agents designed to inhibit

IL-1 or TNF failed to prevent LPS-induced preterm labour in mice (Fidel *et al.*, 1997) but preterm labour can be blocked by indomethacin treatment (Kaga *et al.*, 1996).

Induction by Cytokines

Subcutaneous injections of IL-1 can induce resorption (Silver *et al.*, 1997) or labour in mice (Romero *et al.*, 1991b). Although decidual explants from IL-1-treated mice produce more PGE_2, IL-1 induced fetal death is not mediated by PGE_2 (Silver *et al.*, 1997). Similarly, in rhesus monkeys, intra-amniotic infusion of IL-1β induced amniotic fluid TNF-α, PGE_2 and $PGF_{2\alpha}$, and uterine contractility - but no birth (Baggia *et al.*, 1996). However, as IL-1β and TNF diminished to basal levels, uterine activity also decreased despite continued elevations in amniotic fluid prostaglandins. Intravenous or intra-amniotic IL-10 pre-treatment reduced IL-1β induced uterine contractility (Sadowsky *et al.*, 1999).

4. The Mouse

This thesis will focus on parturition in the mouse with particular emphasis on the cascade of events leading to ethanol-induced preterm birth or normal birth at term. Extensive review of the literature regarding parturition in the mouse reveals an interesting fact. There is very little detail known about the mechanisms underlying term and experimentally-induced preterm birth in mice. While there is a well documented increase in the E:P ratio prior to parturition (McCormack and Greenwald, 1974) at term, only one recent study has examined the temporal changes in progesterone prior to bacteria- or LPS-induced preterm birth (Fidel *et al.*, 1998). There has also been a paucity of data examining the temporal variations in

prostaglandins and cytokines prior to parturition. However, studies using transgenic knockout mice which are deficient in PLA_2 (Uozumi *et al.*, 1997; Bonventre *et al.*, 1997), PGHS (Langenbach *et al.*, 1995; Dinchuk *et al.*, 1995; Morham *et al.*, 1995; Lim *et al.*, 1997; Gross *et al.*, 1998), or the receptor for prostaglandin $F_{2\alpha}$ (Sugimoto *et al.*, 1997) have hinted at an important role for prostaglandins in regulating the normal length of gestation. With respect to cytokines and reproduction, there are numerous studies evaluating the role of TH1/TH2 and pro-/anti-inflammatory cytokines in the establishment and maintenance of pregnancy as well as their role in the context of infection-associated preterm birth (see Chapter 5), but very little study has been conducted on the role of cytokines during normal birth at term. Similarly, the effects of acute alcohol consumption have not been studied in detail in the context of parturition. In subsequent chapters in this thesis, the literature relevant to parturition in the mouse will be address in detail. However, it should be noted that in an effort to provide a more comprehensive perspective on parturition in the mouse, it was often necessary to supplement knowledge deficiencies with data relevant to the rat.

E. RATIONALE

It is clear that steroid hormones, prostaglandins and cytokines play key roles in the physiology of birth whether it occurs at term or preterm. In most species, birth at term is preceded by a decrease in peripheral progesterone levels and an increase in estrogen concentrations. In addition, there are increases in PGE_2 and $PGF_{2\alpha}$ in intrauterine tissues and a potential role for pro- and anti-inflammatory cytokines. However, there is very little detailed information on temporal changes in these mediators leading up to parturition.

Furthermore, the effect that the TH1 and TH2 cytokines play during parturition is at present unknown. Finally, the role of an acute dose of alcohol on events relating to parturition needs further elucidation.

The failure to develop effective strategies for preventing or treating preterm labour has its foundations in the lack of a real understanding of the regulation of parturition in normal and pathophysiological situations. There are several approaches to the study of preterm labour and birth. One way is to address the ultimate origin or initiator of the cascade. However, this is a very difficult task because of the broad spectrum of etiological factors which may contribute to preterm birth. Often, the cause of preterm birth is not identifiable. On the other hand, it is also conceivable to look for common elements between the mechanisms underlying the onset of labour, whether it occurs at term or preterm. Term birth is the result of the complex interactions between several redundant pathways. Preterm birth may simply be an earlier and more compact rendition of the events leading to normal term birth. Alternatively, preterm birth may enlist only a few of the cascades observed during term birth. If the redundancies are abolished with different methods of inducing preterm birth, the remnants may be a mechanism or cascade that is absolutely essential and indispensible for the process of parturition, if it exists at all. As always, it is essential to ask whether intervention should be initiated to prevent or delay preterm labour onset. After all, the cascade was initiated for some reason, whether it is clinically evident or not. Of course, even a delay of a few hours may mean transportation to better equipped facilities.

The use of the mouse model allows a thorough and in-depth examination of the events precipitating birth at term and preterm. By using the mouse model, the temporal profile of several important mediators can be established in the same group of mice and

related to one another. It will then be possible to determine which mechanisms are common and distinct in normal birth at term and birth induced preterm by ethanol administration. The **objective** of this research is to determine changes in the expression of plasma steroid hormones, intrauterine prostaglandins and placental and amniotic fluid cytokines during late gestation and birth in the C57Bl/6J mouse and to compare these changes to those induced by the exposure of these mice to ethanol. The **hypothesis** is that the changes in progesterone, estrogen, PGE_2, $PGF_{2\alpha}$, pro-inflammatory and anti-inflammatory cytokines, and TH1 and TH2 cytokines associated with the onset of normal labour at term are paralleled by the preterm changes induced by ethanol in mice.

CHAPTER II. ETHANOL-INDUCED MOUSE MODEL OF PRETERM BIRTH

A. INTRODUCTION

During the course of unrelated experiments, Salo *et al.* (1996) made the initial observation that acute alcohol consumption late in gestation in the mouse resulted in a shortened gestation. This led to the subsequent development of a mouse model of preterm birth by Cook (1997) and Cook and Randall (1997). In this mouse model, a 6 g/kg acute dose of ethanol given by gavage on gestational day 16 resulted in a 93% incidence of preterm birth. The 6 g/kg dose is teratogenic in this strain and produces blood alcohol levels of 0.379 ± 0.036 g/dL which are completely dissipated from the maternal circulation seven to nine hours post-treatment. Moreover, it was observed that there was a short, critical window of susceptibility to the induction of preterm birth by ethanol and a threshold dose effect rather than a dose-response. This may partially explain the discrepancies in reports of whether acute alcohol exposure affects gestational length since the right dose and consumption pattern has to be consumed at a particular time.

These experiments were conducted to establish the mouse model in our laboratory and to collect tissues in a comprehensive time course in order to study the temporal profile of mediator changes through late gestation until the time of birth. Ultimately, this study will help to elucidate the mechanisms underlying ethanol-induced preterm birth and normal birth at term in the mouse.

B. MATERIALS AND METHODS

1. Mice

Nulliparous C57BL/6J female and male mice (eight to ten weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and were housed in conventional cages in the University of Alberta Health Sciences Laboratory Animal Services facilities. Mice were exposed to a 12 hour light-dark cycle with access to laboratory chow and water *ad libitum*. All protocols were approved by the Animal Care Committee at the University of Alberta.

2. Breeding Procedure

In order to better estimate the time of conception and calculation of gestational length, two females were bred with four males from 09:00 - 10:00 am. Females which showed evidence of potential pregnancy as indicated by the presence of a seminal plug at the end of the breeding session were weighed and housed individually. The day of conception was designated Gestational Day Zero (GD 0). Pregnancy was confirmed if there was significant weight gain by GD 10 or 14. Females were then randomly assigned to either the untreated control group or the ethanol-treated group.

3. Ethanol Administration

Females were fasted for 2 h prior to ethanol administration to ensure there was no interference with the absorption of ethanol from the stomach. At 11:00 am on GD 16, pregnant females were given 6 g/kg ethanol by gavage in a ratio of 0.03 mL of 24.4% ethanol per gram of body weight. Control females received no treatment. Previous studies have

demonstrated that there was no significant difference in the gestational lengths between animals given nutritional isocaloric sucrose and untreated controls (Cook, 1997). Thus, the effects of ethanol on gestational length were not the result of nutritional differences. On this basis, it was decided that untreated females would be an adequate control group both for this study and for future studies involving an alternative method of inducing preterm birth.

4. Tissue Collection

Time Course

Pregnant females were euthanized at various time points (see Figure II-1) by decapitation for tissue harvesting. More specifically, tissues from untreated control mice were collected on GD 14.0, 15.0, and every twelve hours from GD 16.0 until the onset of parturition at term with additional time points representing six hour intervals during the last twenty-four hours prior to parturition. The last time point, parturition, is referred to as term birth. Thus, for the untreated controls, there are a total of eleven groups with n=8-18 in each group. Tissues were harvested from ethanol-treated mice every six hours from GD 16.0 until the onset of preterm parturition. The last time point in this time course, ethanol-induced parturition, is referred to as preterm birth. In the ethanol-treated mice, there are a total of seven groups with n=7-17 in each group. In both instances, parturition was defined as the delivery of the first pup when the females were actively labouring. Labour was not visibly protracted relative to controls. In the groups which delivered either at term or preterm, the gestational length was calculated and recorded. Gestational lengths were expressed in days.

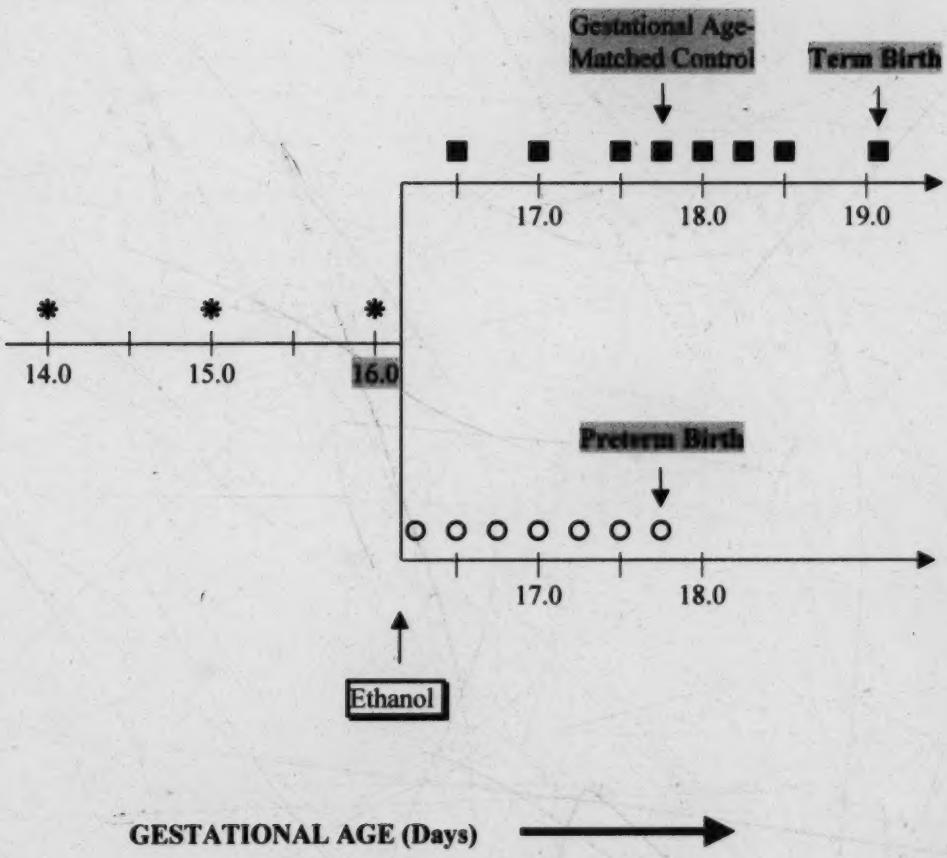

Figure II-1. Tissue and plasma collection time points. (✱ = Common to Untreated Control and Ethanol Treated Mice, ■ = Untreated Control Time Course, O = Ethanol Time Course).

Key Points

In certain instances, only four key time points from the full time courses were examined. These key time points include the following: (1) untreated control GD 16.0, a group common to both the ethanol-treated and untreated control groups, (2) untreated control GD 17.75, the gestational age-matched control for preterm birth, (3) preterm birth, and (4) term birth.

Tissue Harvesting

Following the decapitation of each mouse, trunk blood was collected into a 1.5 mL eppendorf microcentrifuge tube containing a drop of heparin. The blood was centrifuged at 14,000 x *g* for 10 min at 4°C and the plasma was collected and stored at –20°C. In addition, pups were delivered by caesarian section. Briefly, a midline incision was made and the uterus and ovaries were exposed, rinsed with saline (0.9% NaCl) and dissected out. The ovaries were cleared of the surrounding fatty tissues and snap frozen in liquid nitrogen. A longitudinal cut was made in the uterus along its entire length to expose the individual placentas, amniotic sacs and fetuses. These fetal units were bluntly dissected away from the uterus and the uterus was then snap frozen in liquid nitrogen. Whenever possible, amniotic fluid was collected using a sterile needle and syringe and stored at –20°C. The fetal membranes were separated from the placentas as thoroughly as possible using scissors and both tissues were snap frozen in liquid nitrogen. Finally, the pups were euthanized with 2 µL of Euthanyl. The number of pups and average litter weight were noted. All snap frozen tissue was stored at –80°C in small plastic scintillation vials.

5. Statistical Analysis

All results are expressed as the mean ± the SEM. In this thesis, statistical analysis was performed using SigmaStat (version 2.0, SPSS Inc.). A combination of one-way analysis of variance (ANOVA) and Student's t-tests were used to analyze the data since the existence of missing time points between the untreated controls and ethanol-treated time courses prevented the use of a two-way ANOVA. The effect of ethanol on gestational length was analyzed by Student's t-test. In order to detect changes in litter size or pup weight with advancing gestational age, a one-way ANOVA was conducted for the untreated control time course with post-hoc analyses by Tukey's test as required. Since only two time points (GD 16.0 and Preterm Birth) were examined in the ethanol-treated time course, a Student's t-test was used to examine the effect of gestational age on cytokine levels. Further analyses with Student's t-tests were utilized to examine differences between the untreated (control) and ethanol-treated (experimental) groups at key time points. These included comparisons between term birth and preterm birth groups as well as comparisons between preterm birth and its gestational age-matched control. A statistical significance level of $p \leq 0.05$ was chosen.

C. RESULTS

The effect of ethanol on gestational length is illustrated in Figure II-2. Untreated control animals (n=18) had gestational lengths of 19.12 ± 0.09 days whereas ethanol administration on GD 16.10 (n=24) significantly shortened gestation to 17.75 ± 0.09 days ($t_{40}=10.91$, $p<0.001$). Pups born to ethanol-treated females were alive at the time of birth but

died shortly afterwards. In contrast, litters born to untreated control females survived beyond the perinatal period. In this thesis, the untreated control GD 17.75 group will serve as the gestational age-matched control for the preterm birth group unless specified otherwise.

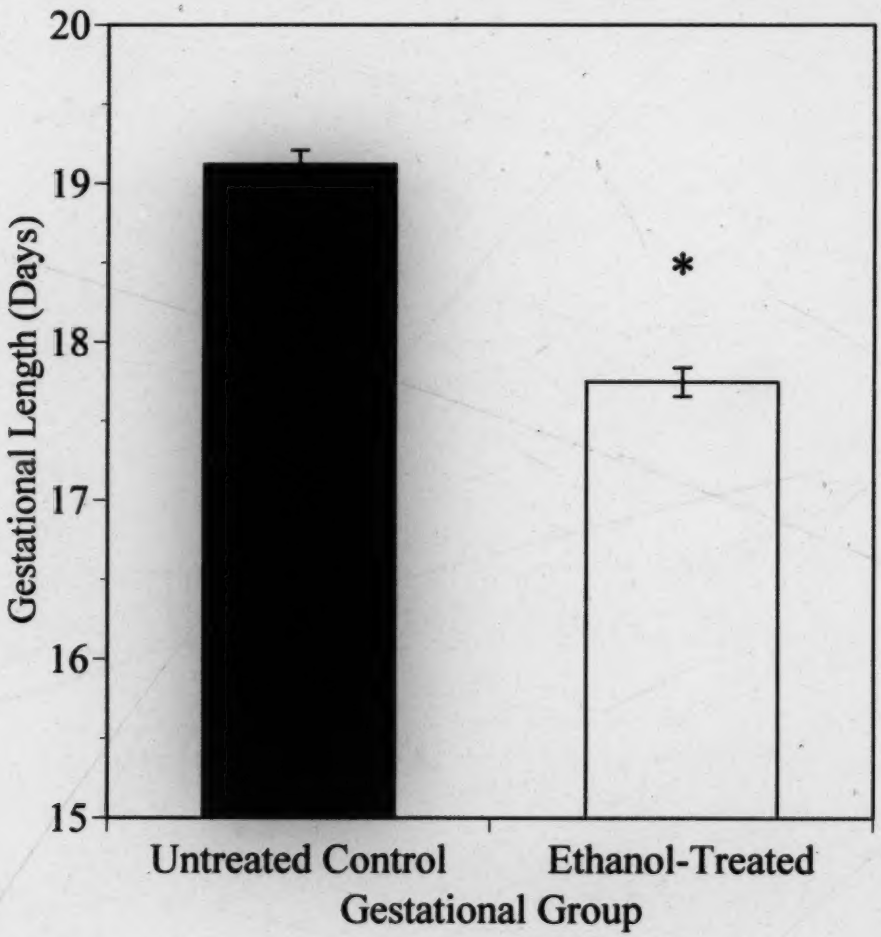

Figure II-2. The effect of ethanol on gestational length (mean ± SEM) in the C57BL/6J mouse. The * represents a significant difference ($p<0.05$) between the ethanol-treated (n=24) and untreated control (n=18) groups.

The average number of pups in a litter and their average individual masses at birth are summarized in Table II-1. There was no effect of gestational age or ethanol treatment on litter size. In contrast, gestational age had a significant effect on the average pup mass in untreated controls ($F(2,44)=222.1$, $p<0.001$) and post-hoc analysis showed that the average pup mass increased with increasing gestational age. Similarly, pup weight was significantly elevated in the preterm birth group when compared to GD 16.0 ($t_{25}=6.84$, $p<0.001$). Furthermore, the average pup mass was smaller in the preterm birth group when compared to either the term birth group ($t_{27}=18.08$, $p<0.001$) or the untreated, gestational age-matched control ($t_{29}=4.67$, $p<0.001$).

Table II-1. The Effect of Gestational Age and Ethanol on Litter Size and Average Pup Mass (mean ± SEM) in the C57BL/6J Mouse.

Mouse Group	Gestational Age		
	GD 16.00	GD 17.75	GD 19.10
Litter Size			
Untreated Control	7.3 ± 0.5 (n=22)	7.7 ± 0.5 (n=17)	7.7 ± 0.4 (n=17)
Ethanol-Treated	7.3 ± 0.5 (n=22)	7.7 ± 0.4 (n=19)	-
Average Pup Mass (g/pup)			
Untreated Control	0.53 ± 0.02 [a] (n=13)	0.98 ± 0.04 [b] (n=17)	1.38 ± 0.02 [c] (n=15)
Ethanol-Treated	0.53 ± 0.02 [a] (n=13)	0.76 ± 0.03 [b*] (n=14)	-

*Different letters represent a statistically significant difference ($p\leq0.001$) with increasing gestational age ($p\leq0.001$). The * denotes a statistically significant difference ($p\leq0.001$) between the ethanol-treated and untreated control groups on GD 17.75 and during birth.*

D. DISCUSSION

The use of mice as an animal model for the study of preterm birth is controversial for several reasons (Oshiro *et al.*, 1994). Ideally, the animal model should be as physiologically equivalent to the human as possible. Although the physiology of mice and humans share many similarities, they also possess very distinct differences which will be addressed below. In addition, spontaneous preterm birth occurs very rarely in animals (only in humans) and must be experimentally induced. Consequently, careful consideration must be given to whether the observations are true, relevant effects or just adverse side effects from the experimental manipulation. Unfortunately, ethical and moral considerations render extensive, in-depth experimentation in humans impossible. Animal models remain the only alternative for studying the mechanisms which precipitate normal and pathophysiological birth. While the data obtained may prove relevant and applicable to humans, it will always be requisite to use caution in interpreting the data and extrapolating it to humans.

Similarities and Benefits

The use of mice is both conducive to controlled studies and ethically more acceptable in the development of new pharmacotherapies. Of the various benefits that can be attributed to murine models, the ease of handling, low maintenance, low expenses and relatively short gestational length are prime considerations. As for the relevance of physiological events in mice to humans, the mouse is an ideal animal model by virtue of several reasons. Humans and mice share a similar haemochorial placenta (Damsky *et al.*, 1993) with the same number of tissue layers separating the maternal and fetal circulations and the presence of one disc-

shaped placenta for each fetus (Morriss and Boyd, 1988). The immune system is very well characterized which translates into an increased availability of commercially available mouse-specific antibodies and probes (Hirsch *et al.*, 1995). Moreover, the mouse is the best model for conducting transgenic, knock-out experiments in which the ability to express specific moieties is selectively deleted. However, transgenic models must be interpreted with caution because of the intrinsic redundancy wired into physiological systems (Challis, 1997).

Differences and Limitations

On the other hand, the mouse model is not without limitations. Most noticeably, mice are polytoccous and produce their litters in bicornuate uteri. Accordingly, it is possible that birth may be initiated by one, all or a proportion of the fetuses. In addition, there must be an inherent synchrony between fetuses since nonviable littermates are not preferentially expelled, and the fetuses are delivered in an orderly manner at term (one horn at a time). Furthermore, the placentas of mice possess labyrinthine vascular arrangements whereas the human placenta is villous (Morriss and Boyd, 1988). With respect to the placental interface function, the mouse possesses an additional surface of exchange in its yolk sac. Unlike human females, the endometrial layer of the mouse uterus does not completely decidualize. Rather, decidualization only occurs in the vicinity of the implanted embryo (Aplin, 1991). More importantly, the mouse is dependent upon the corpus luteum for progesterone production in order to maintain pregnancy and degeneration of the corpus luteum during luteolysis creates a withdrawal of progesterone at the end of pregnancy (Csapo, 1956). The subsequent increase in the maternal plasma estrogen to progesterone ratio observed in mice is thought to be involved in labour initiation (McCormack and Greenwald, 1974; Murr *et al.*,

1974). Although humans initially rely upon the corpus luteum as its source of progesterone, progesterone production shifts to the placenta approximately ten weeks into gestation (Guyton and Hall, 1996) and the existence of an increase in the estrogen to progesterone ratio remains controversial.

All of these theories focus on the hemochorial placenta, the primary interface between maternal and fetal tissues in humans. More specifically, the trophoblast which is strategically located within this intimate juxtaposition of maternal and fetal cells. For a review of placentation in humans, please refer to Aplin (1991). The murine hemochorial placenta possesses several distinct features. The Reichert membrane is an effective barrier to the passage of cells and immunoglobulins (Herrera-Gonzalez and Dresser 1993). According to Hunziker and Wegmann (1986), the giant cells, spongiotrophoblast and yolk sac are in direct contact with maternal decidual tissue and the labyrinthine trophoblasts are bathed in maternal blood.

Effect of Alcohol

Mice are less sensitive to the effects of alcohol than humans (Carson and Pruett, 1996). Consequently, in order to elicit the same behavioural changes typical of humans, higher blood alcohol levels than those expected in humans are required (Carson and Pruett, 1996). Behavioural changes may be better basis for comparison rather than doses or blood alcohol levels (Majchrowicz, 1975) for several reasons: (1) In small animals, dose responses are better predicted by body surface area (Tichy *et al.*, 1985). (2) Mice can tolerate and survive much higher blood alcohol levels than humans (~900 vs. 400 mg/dL) (Finn *et al.*, 1989; Rall, 1990) but there have been records of humans attaining similar blood alcohol

levels (Mello and Mendelson, 1970; Dunbar *et al.*, 1982). (3) The clearance of ethanol is also more rapid in mice (Wu *et al.*, 1994) than in humans (Andrews and Snyder, 1991). It is unknown whether peak concentrations or duration of a certain concentration for a certain time period is more important (Wu *et al.*, 1994).

With respect to the adverse effects of alcohol, female mice treated with ethanol lose their righting reflex for several hours following ethanol treatment and remain lethargic for the next twenty-four hours. However, the females do recover and the pups are alive at the time of birth. The lower average pup mass observed in ethanol-treated mice during birth when compared to its gestational age-matched control may be a reflection of the detrimental effect of alcohol on fetal growth. Ethanol is associated with a reduction in amino acid transport across the placenta as well as transient fetal hypoxia due to impairment of umbilical circulation (reviewed in Randall *et al.*, 1987). In addition, ethanol may exert direct effects on fetal growth since ethanol is transferred across the placenta without impediment and the amniotic fluid acts as a ethanol reservoir to prolong ethanol exposure beyond the point when maternal blood alcohol levels have returned to normal (reviewed in Smith *et al.*, 1991). Conversely, this difference may be due to the inability of the females to consume food and water during their time of intoxication.

A binge drinking model in non-pregnant female mice has been recently described using 5.0 to 7.0 g/kg ethanol administered by gavage (Han *et al.*, 1993; Wu *et al.*, 1994). At these doses, behavioural changes (ranging from mild ataxia to unresponsiveness) similar to those observed in humans after an episode of heavy drinking were observed. In this model, a single dose of ethanol (5-7 g/kg) to non-pregnant female mice (B6C3F1) resulted in blood alcohol levels ranging from 0.2% to 0.5% and at a dose of 6 g/kg, all of the ethanol was

cleared 8 hours after administration. Acute ethanol produces a stress response (Thiagarajan *et al.*, 1989) which is characterized by a 10-fold increase in endogenous levels of glucocorticoids (corticosterone) (Han *et al.*, 1993) but no gross abnormalities or histopathological changes were observed in the liver, kidney, brain or lungs. In order to address concerns that the effects of ethanol were secondary to drastic alterations in various physiological processes, Carson and Pruett (1996) examined general clinical chemical parameters at various times after ethanol treatment and found no biologically relevant changes.

E. CONCLUSIONS

Acute alcohol exposure on GD 16.1 in C56BL/6J mice consistently induces preterm birth which occurs on GD 17.75 ± 0.1 days. Normal term gestation in this strain occurs on GD 19.1 ± 0.1 days. The use of ethanol and the use of mice in our studies is not without controversy. Foremost is the lack of a clear clinical relevance of acute ethanol on preterm parturition in humans. Furthermore, the adverse effects of ethanol on pregnancy and parturition can be completely avoided through abstinence and educational awareness. Nevertheless, ethanol is an independent stimulus which can reliably induce preterm birth in mice. This feature permits the performance of experiments designed to evaluate gestational age-related changes in detail. Thus, this mouse model will be an invaluable tool for dissecting out the similarities and differences in the temporal mediator changes precipitating preterm birth and term birth in the mouse.

CHAPTER III. SEX STEROIDS

A. INTRODUCTION

Term Birth

In mice and rats, normal birth at term is clearly associated with a decrease in maternal peripheral progesterone levels and a concomitant increase in peripheral estrogen levels, both of which contribute to an increase in the estrogen to progesterone (E:P) ratio (McCormack and Greenwald, 1974; Thorburn and Challis, 1979). The loss of progesterone dominance is important in relieving the suppression of myometrial activity whereas the emergence of estrogen dominance up-regulates gap junction formation and facilitates the action of other uterotonic agents by regulating their synthesis, metabolism and receptor expression.

Preterm Birth

Progesterone withdrawal appears to be an important prerequisite of both full term birth and preterm birth in mice. A recent study by Fidel *et al.* (1998) showed that systemic LPS treatment resulted in serum progesterone levels that were consistently lower than controls from 1 h following LPS-treatment until the time of LPS-induced preterm birth. Thus, progesterone withdrawal preceded LPS-induced preterm birth in mice. Furthermore, preterm birth can be induced by a form of progesterone withdrawal involving the administration of the progesterone receptor antagonist, RU486, in mice (Dudley *et al.*, 1996) and rats (Fang *et al.*, 1997). In rats and mice, ovariectomy, or the removal of the main endogenous source of progesterone, also leads to preterm birth (Sharma and Bulmer, 1983).

The levels of estrogen have not been studied in the context of LPS-induced preterm birth in mice. However, blocking of estrogen action through the use of tamoxifen, an estrogen receptor antagonist, late in gestation in the rat significantly prolongs gestation beyond term by 24 h (Fang *et al.*, 1996). In these rats, the increase in the E:P ratio was attenuated compared to controls. Moreover, serum estradiol levels and uterine ER remained low whereas progesterone withdrawal was not influenced. However, birth did occur despite continued tamoxifen administration thereby underlining the relative importance of progesterone withdrawal over estrogen elevation.

Purpose

These studies were conducted in order to profile the temporal changes in plasma estrogen levels in the context of preterm birth induced by acute exposure to ethanol in mice and to compare these changes to the events occurring during normal full term gestation. Furthermore, the relationship between estrogen and progesterone was also evaluated using progesterone measurements made by other members of this laboratory. It was hypothesized that an increase in the E:P ratio occurs at both term birth and ethanol-induced preterm birth.

B. MATERIALS AND METHODS

1. Materials

Diethyl Ether (anhydrous) (VWR Canlab, Mississauga, Ontario)

DPC Double Antibody ^{125}I Estradiol Kit (InterMedico, Markham, Ontario, Cat. No. KE2D1).

2. Plasma 17β-Estradiol

Plasma 17β-estradiol levels were measured in the untreated control (n= 6-13/time point) and ethanol-treated time courses (n = 5-16/time point).

Extraction From Plasma

The extraction of 17β-estradiol from maternal plasma was performed in order to measure the biologically active, unconjugated estradiol present and to remove any substances which may interfere with the radioimmunoassay. 17β-estradiol was extracted from plasma as suggested by the instructions included with the ^{125}I-estradiol double antibody kit. Briefly, 100 µL of plasma was extracted in glass vials with teflon-lined caps using 800 µL of anhydrous diethyl ether. Blanks were prepared with diethyl ether alone. Vials were capped and thoroughly vortexed three times for 30 sec each time before centrifugation at 2,300 x *g* for 5 min at room temperature. The lower aqueous phase was frozen and the organic phase was decanted to a glass test tube. The diethyl ether was evaporated to dryness under a gentle stream of nitrogen. Extracts were reconstituted in 200 µL of the zero calibrator supplied with the kit and thoroughly vortexed.

Radioimmunoassay

The 17β-estradiol radioimmunoassay (RIA) was performed as per the instructions included with the kit. Briefly, a standard curve was prepared in duplicate using 200 µL of pre-diluted standards ranging from 5 to 500 pg/mL in 12x75 mm polypropylene test tubes. An additional 3 pg/mL standard was diluted from the 5 pg/mL standard using the supplied zero calibrator. Non-specific binding (NSB) tubes and maximal binding (Bmax) tubes were

also prepared using 200 μL of zero calibrator. No zero calibrator was added to total count (T) tubes. Frozen aliquots of quality controls were diluted and run in duplicate in each assay. Extracted samples were reconstituted in 100 μL of zero calibrator, transferred to the polypropylene test tubes and an additional 100 μL of zero calibrator was added. Due to limitations in the amount of plasma available, only one tube for each sample was assayed. Anti-estradiol antibody (100 μL) raised in rabbits and supplied with the kit was added to each tube except for the NSB tubes. All tubes were vortexed and incubated at room temperature for 2 h. ^{125}I- labelled synthetic 17β-estradiol in liquid form was then added in a volume of 100 μL to every tube to compete with the plasma estradiol for antibody sites. All tubes were vortexed and incubated at room temperature for 1 h. In order to separate bound from free antibody, 1 mL of cold precipitating solution (goat anti-rabbit gamma globulin second antibody and dilute polyethylene glycol in saline, supplied with kit) was added to each tube except T tubes, vortexed and incubated for 10 min at room temperature. Finally, tubes were centrifuged at 4°C for 30 min (2,300 x *g*) to pellet the antibody-bound fraction. The supernatant was aspirated, the tubes were inverted and dried overnight before the pellets were counted in a Gamma-8000 counter for 1 min to obtain the counts per minute (CPMs). Sample values were corrected for recovery and blank values were subtracted from them. Results were expressed in pg 17β-estradiol/mL.

In all RIAs presented in this thesis, T tubes represent the total amount of radioactivity present in the test tubes after completion of the assay, NSB tubes are a measure of the background and non-specific binding of estradiol to substances other than the antibody, and Bmax tubes show the maximum amount of steroid hormone or prostaglandin that can bind

the antibody in the assay. In order to determine the concentrations of the unknown samples, the percentage bound is calculated from the assay CPM's using the following formula:

$$\text{Percentage Bound} = [(\text{Sample} - \text{NSB}) / (\text{Bmax} - \text{NSB})] \times 100\%$$

A standard curve was then generated on a logit-log graph by plotting percent bound (y-axis) versus concentration (x-axis) and sample concentrations could then be interpolated from a best-fit line drawn on the graph.

Characterization of Extraction and Radioimmunoassay

Extraction Recovery

Rather than determining a specific recovery value for each sample, an average recovery value was calculated and applied to each sample and blank. Recovery total count tubes (100 µL of 1:10 diluted ^{125}I-17β-estradiol), background tubes (100 µL PBS, 100 µL plasma, 800 µL diethyl ether) and sample tubes (100 µL of 1:10 diluted ^{125}I-17β-estradiol, 100 µL plasma, 800 µL diethyl ether) were prepared in triplicate. The average recovery value was estimated at 72.9% and was calculated using the following formula:

$$\text{Recovery} = (\text{Sample} - \text{Background})/(\text{Total Counts} - \text{Background}) \times 100\%$$

Accuracy, Precision, Sensitivity and Specificity

In order to maximize the confidence in the values obtained from assays presented in this thesis, several parameters including accuracy, precision, sensitivity and specificity were determined whenever possible. (1) Assay accuracy is the closeness of the measured value obtained from the assay to its true value. Indices of the 17β-estradiol radioimmunoassay accuracy are listed in Table III-1 and were determined by measuring known quantities of

17β-estradiol in quadruplicate in an assay. (2) The precision of an assay is the closeness of repeated measurements of the same quantity. The precision within a given assay is measured by an intra-assay coefficient of variation (COV) whereas the variability between assays performed at different times is quantified by an inter-assay COV. Variation between assays reflect differences when assays were done at different times using different reagents and reagents which may have degraded over time in storage. The precision of the 17β-estradiol radioimmunoassay is summarized in Table III-1 and was obtained from three separate assays consisting of T, NSB and Bmax tubes in duplicate, duplicate standard curve and duplicate quality controls representing a middle pool (30 pg/mL) and a low pool (8 pg/mL).

$$\text{Intra-assay COV} = \frac{\text{(SD of replicates of Quality Controls)}}{\text{(Mean of replicates of Quality Controls)}} \times 100\%$$

$$\text{Inter-assay COV} = \frac{\text{(SD of replicate standard curve averages of Quality Controls)}}{\text{(Mean of replicate standard curve averages of Quality Controls)}} \times 100\%$$

Table III-1. 17β-Estradiol Radioimmunoassay Accuracy and Precision

Concentration Added (pg/mL)	Concentration Observed (pg/mL)	Intra-Assay Coefficient of Variation (%)	Inter-Assay Coefficient of Variation (%)
30	26.7 ± 3.5	9.3	12.9
8	4.3 ± 0.4	16.4	16.5

n=4 per group

(3) The sensitivity of the assay represents the limit of detection of the assay which by definition is the amount of the steroid which is significantly different from zero. In order to

determine this, it is necessary to determine the mean and standard deviation in the counts per minute (CPM's) of several replicates of Bmax. The formula for calculating sensitivity is as follows:

$$\text{Sensitivity} = \text{Mean CPM of Bmax replicates} \pm 2\ \text{SD}$$

The corresponding 17β-estradiol value at that CPM represents the limit of detection of the assay. Therefore, the sensitivity of the 17β-estradiol RIA was 0.8 pg/mL. (4) Finally, the specificity of the estradiol antibody was determined by InterMedico and had a reported 12.5% cross-reactivity with estrone. There was no cross-reactivity (<10%) with other estrogens.

Additional Assay Validations

In order to ensure that this assay could determine relative differences between samples, three different concentrations of 17β-estradiol (8 pg/mL, 20 pg/mL and 100 pg/mL) were added to samples and blanks during the assay. The results were then graphed (Figure III-1) and the slopes of best-fit lines for the blanks and samples were compared. The slopes (blanks = 0.86 and samples = 0.86) were similar thereby adding more confidence to the measurements arising from this RIA. Furthermore, different dilutions (1:1, 1:2, 1:4) of plasma were assayed and 17β-estradiol levels measured from these samples were consistent with the dilutions.

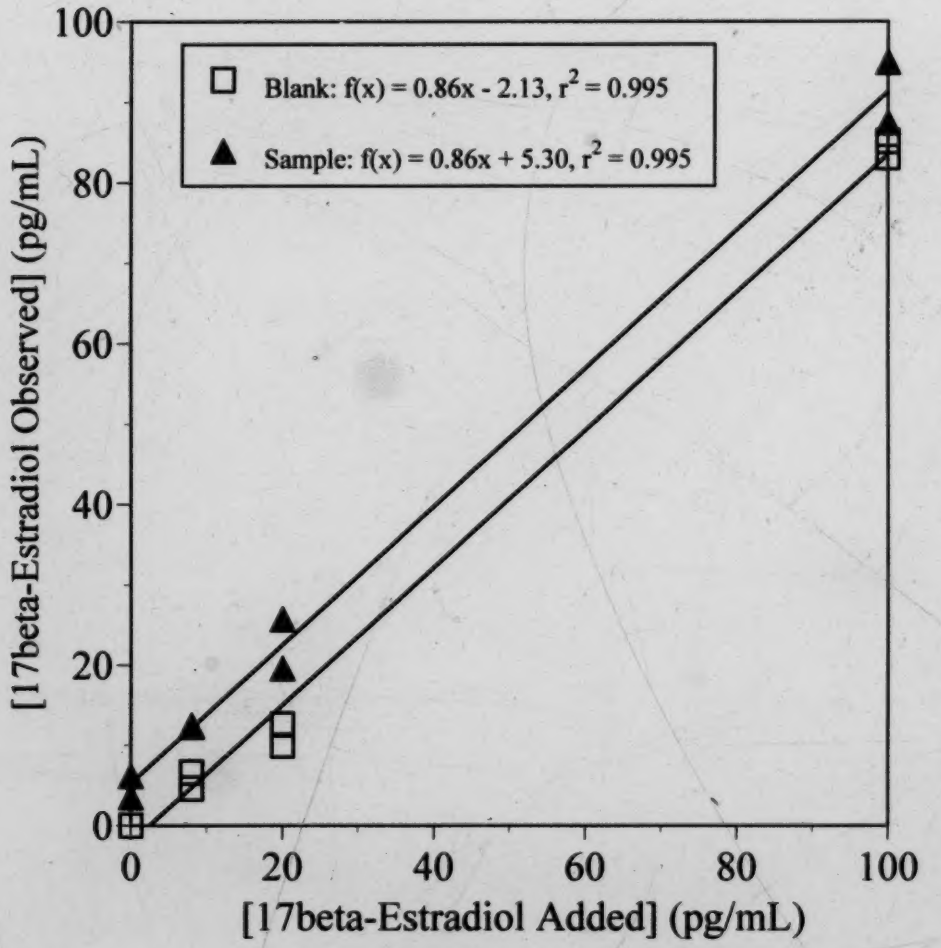

Figure III-1. 17β-estradiol additional assay validation. The observed values of 17β-estradiol obtained by RIA were plotted against the known concentrations of 17β-estradiol which were added to samples and blanks prior to assay.

3. Statistical Analysis

All results are expressed as the mean ± SEM. In order to detect changes with advancing gestational age, separate one-way analysis of variance (ANOVAs) (SigmaStat version 2.0, SPSS Inc.) were conducted for the untreated control and ethanol-treated time courses with post-hoc analyses by Tukey's test as required. Further analyses with Student's t-tests were utilized to examine differences between the untreated (control) and ethanol-treated (experimental) groups at key time points. These included comparisons between term birth and preterm birth groups as well as comparisons between preterm birth and its gestational age-matched control. A statistical level of significance of $p \leq 0.05$ was chosen.

C. RESULTS

1. Plasma 17β-Estradiol

Changes in plasma 17β-estradiol levels over gestation in control and ethanol-treated mice are depicted in Figure III-2. In the control group, there is a significant effect of gestational age on plasma 17β-estradiol levels ($F(8,69)=7.162$, $p<0.001$). Estradiol levels are low on GD 16.0 (2.42 ± 0.6 pg/mL) and are elevated during term parturition (11.9 ± 1.9 pg/mL, $p<0.05$). In contrast, there is no effect of gestational age on plasma estradiol levels in ethanol treated females ($F(96,54)=0.984$, $p=0.447$). Figure III-3 illustrates the comparison between preterm and term birth. A student t-test reveals that term parturition is associated with significantly higher levels ($t_{27}=3.96$, $p<0.001$) of plasma 17β-estradiol (11.9 ± 1.9 pg/mL) than preterm parturition (4.4 ± 0.8 pg/mL). Furthermore, in comparison to its untreated gestational age-matched control (GD 17.75), 17β-estradiol levels in the females

undergoing preterm parturition are significantly lower (4.4 ± 0.8 pg/mL vs. 8.2 ± 1.4 pg/mL, t_{22}=2.50, p=0.02) and this is shown in Figure III-4.

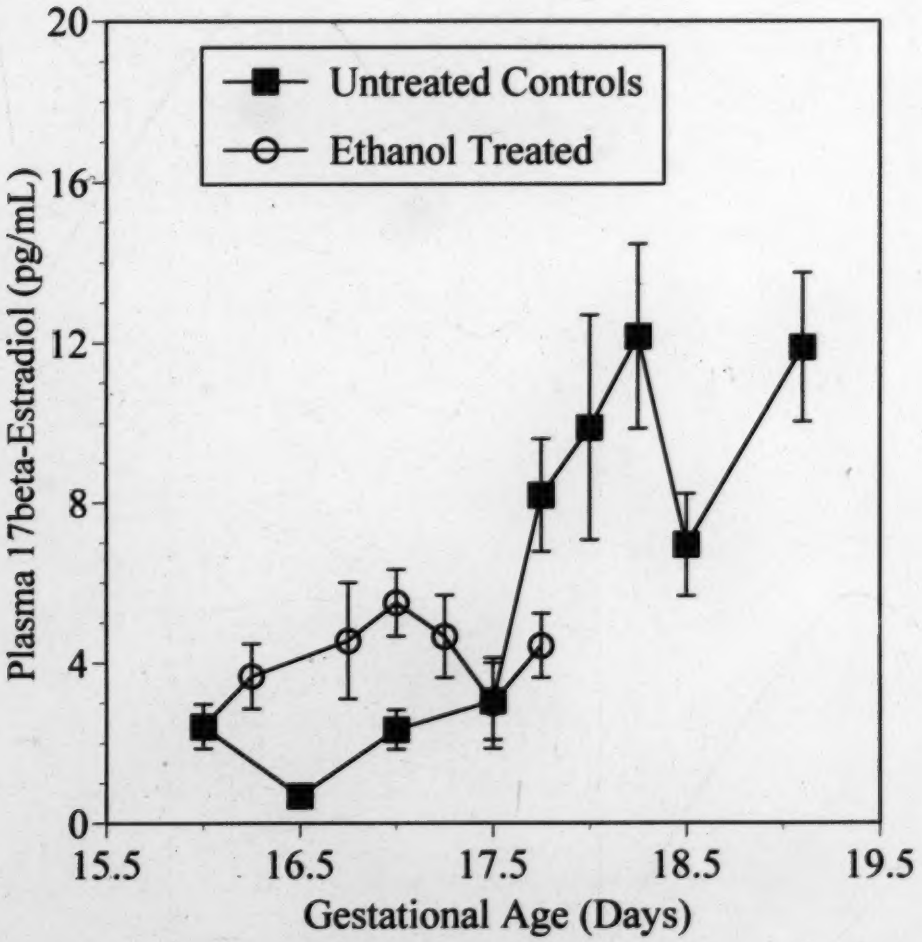

Figure III-2. The effect of gestational age on plasma 17β-estradiol concentrations (mean ± SEM) in ethanol-treated and untreated controls. There was a significant effect of gestational age (p≤0.001) in the untreated controls (n=6-13 per time point) but not in the ethanol-treated mice (p>0.05) (n=5-16 per time point).

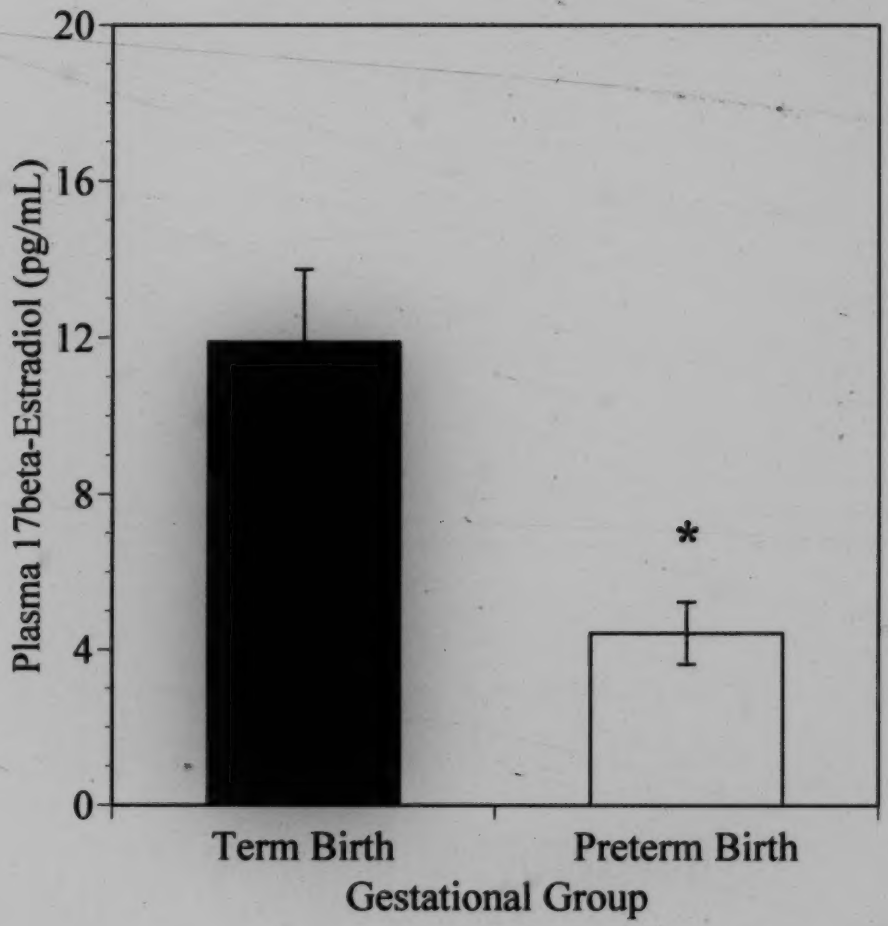

Figure III-3. The effect of ethanol on plasma 17β-estradiol concentrations (mean ± SEM) during parturition in untreated controls (n=13) and ethanol-treated mice (n=16). The * denotes a significant difference ($p \leq 0.05$).

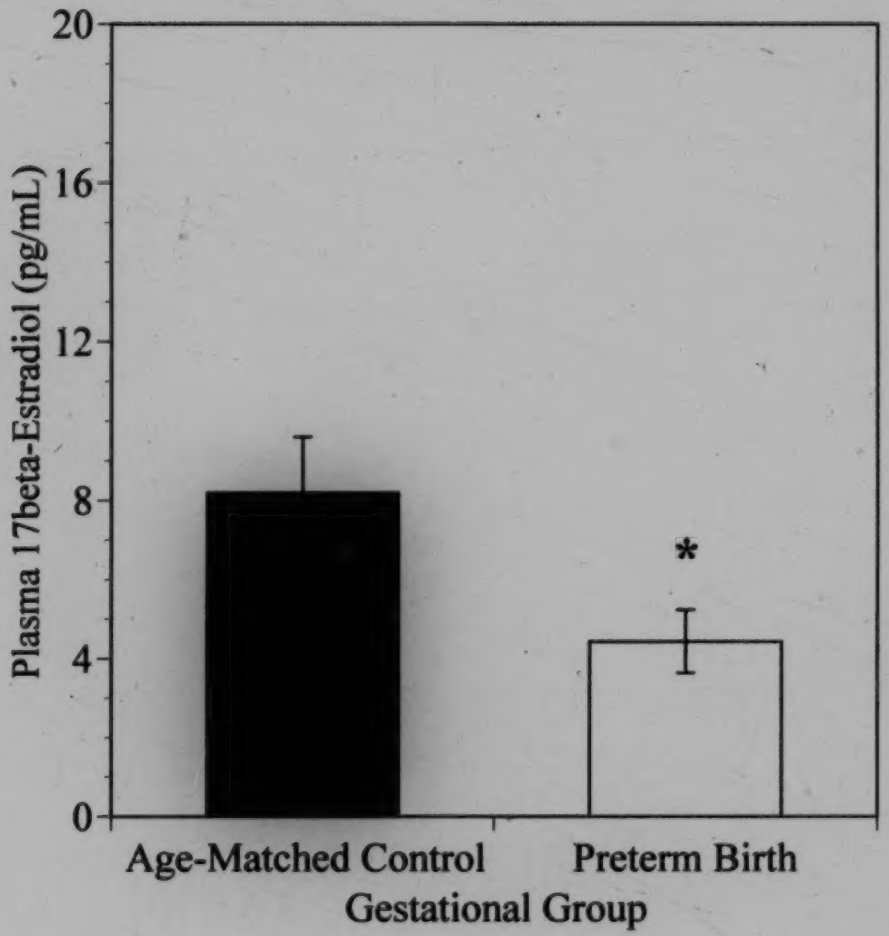

Figure III-4. The effect of ethanol on plasma 17β-estradiol concentrations (mean ± SEM) on GD 17.75 in untreated controls (n=8) and ethanol-treated mice (n=16). 17β-estradiol levels were significantly lower during preterm birth ($p \leq 0.05$).

2. Plasma Estrogen to Progesterone Ratio

In order to calculate the estrogen to progesterone ratio in our mice, plasma progesterone levels were measured by Cook *et al.* (in press) in the same laboratory and are utilized in this thesis with the authors' consent.

Approximations of the estrogen to progesterone ratio at several time points were calculated and are summarized in Table III-2. These ratios were calculated by dividing the average plasma 17β-estradiol levels by the average plasma progesterone levels in equivalent units in each group. No statistical analysis could be performed on these ratios since measures of variability were not available. Ideally, the ratio would be calculated for each individual mouse which would make possible the calculation of variation around the mean. However, plasma quantities were often limiting and prevented the measurement of both estradiol and progesterone from the same mouse.

Table III-2. The Effect of Gestational Age on the Plasma 17β-Estradiol to Progesterone Ratio in Untreated Control and Ethanol-Treated Mice.

Mouse Group	17β-Estradiol to Progesterone Ratio ($\times 10^{-5}$)		
	GD 16.00	GD 17.75	GD 19.1
Untreated Control	4.2	17.1	306.4
Ethanol-Treated	4.2	1265.7	-

For each group, the average plasma 17β-estradiol levels were divided by the average plasma progesterone levels (obtained from Cook et al., (in press)) in equivalent units (pg/mL) to obtain an approximation of the estrogen to progesterone ratio.

D. DISCUSSION

Progesterone withdrawal is a prerequisite of both term birth and ethanol-induced preterm birth in the mouse. According to Cook *et al.* (in press), plasma progesterone levels in the untreated control time course were maximal on GD 15.00, decreased gradually and were significantly lower by GD 18.00 until parturition at term. In contrast, a statistically significant decrease in plasma progesterone levels in ethanol-treated females was evident by GD 17.50 and was at a minimum during preterm labour. Furthermore, preterm birth was characterized by significantly lower plasma progesterone levels in comparison to term birth or its gestational age-matched control. Thus ethanol exposure resulted in a more rapid decline in plasma progesterone levels. In the experiments presented herein, estradiol, in contrast, was observed to increase in plasma prior to parturition at term but this increase was abrogated by acute ethanol exposure. The slight elevation of 17β-estradiol concentrations in the ethanol-treated mice relative to untreated controls during the period of time from GD 16.0 until GD 17.5 may be a consequence of the dehydration of the females and potential concentration of plasma constituents. However, the statistical analysis conducted in this study did not directly address whether there were differences between the ethanol-treated mice and untreated controls at these specific time points. Levels of estradiol and progesterone observed in our mice were in agreement with previously published values (McCormack and Greenwald, 1974; Murr *et al.*, 1974; Fidel *et al.*, 1998). It should also be noted that the levels of free estradiol measured in this study only represent a very small fraction (<5%) of the total estradiol present in the blood which includes conjugated or bound forms. In addition, these levels can be influenced by variations in production, cellular uptake, and clearance rates.

Estrogen to Progesterone Ratio

Despite this difference in the mechanisms underlying preterm and term birth, there is still an overall increase in the estrogen to progesterone level. In the untreated controls, an increase in the E:P ratio is achieved by a decrease in progesterone levels and a concomitant increase in estrogen levels. In ethanol-treated mice, the increase in the E:P ratio is associated solely with a withdrawal of progesterone since estrogen levels do not change. This is not the only time that birth is associated with no change in estrogen levels. Preterm birth can be induced by ovariectomy which removes the main endogenous source of both progesterone and estrogens during pregnancy. Also, the tamoxifen-induced delay in parturition observed in rats is postulated to result from an estrogen-independent increase in prostaglandins which initiates a positive feedback circle involving oxytocin and oxytocin receptor. These mediators can bring about parturition despite depressed estrogen levels in the periphery (Fang, 1996).

Steroidogenesis

During pregnancy, ovarian corpora lutea are the primary sites of steroidogenesis in the rat and mouse (Csapo, 1956) with a minor steroidogenic contribution from the placenta (Soares and Talamantes, 1982, Benbow and Waddell, 1995). In the absence of corpora lutea, placental progesterone production is not sufficient to maintain pregnancy (Murr *et al.*, 1974). Progesterone is synthesized from the abundant stores of cholesterol in the luteal cells. The placentas are essential sources of androgens such as androstenedione which serve as precursors for aromatization and estrogen synthesis by the ovary (Gibori and Sridaran, 1981). Studies by Akinola *et al.* (1997) clearly profile the expression of 17β-HSD Type 1 mRNA, P450 aromatase mRNA and 20α-HSD mRNA in pregnant rat ovaries. 17β-HSD Type 1 was

expressed constitutively. In addition, P450 aromatase increased during late gestation and disappeared prior to parturition whereas 20α-HSD was high early in gestation, decreased with advancing gestation and then increased prior to parturition. The opposite patterns of expression of P450 aromatase and 20α-HSD suggests a local modulation of E:P ratio within the pregnant rat ovary. As expected, P450 aromatase was localized to the corpora lutea but contrary to expectations, 17β-HSD type 1 was localized only to nonluteal granulosa cells and was virtually absent from corpora lutea. This remains unexplained. Gibori and Sridaran (1981) distinguished between luteal and nonluteal ovarian tissues. Luteal tissue estradiol production was controlled by the placentas and was important for maintaining progesterone synthesis. In contrast, nonluteal estradiol synthesis was regulated by the pituitary and prior to parturition in the rat, the estradiol and androgen concentrations in nonluteal tissue were increased which may contribute to the circulating increase in the E:P ratio.

During the first half of pregnancy in rats and mice, steroidogenesis in the corpora lutea is regulated by pituitary luteotropins such as prolactin and luteinizing hormone (LH) (Akinola *et al.*, 1997). Approximately halfway through pregnancy, this regulatory role is transferrred to the placenta (rat placental lactogen) (McCormack and Greenwald, 1974). During this transition, there is an increase in circulating androgens which serves as precursors for estrogen synthesis which can then support and sustain luteal progesterone production (Gibori and Keyes, 1978). Although LH is ineffective during the second half of pregnancy, a functional role for LH in regulating steroidogenesis re-emerges towards the end of parturition as described below (Stocco and Deis, 1996).

Luteolysis

At term, regression of the corpus luteum (luteolysis) occurs leading to an increase in the E:P ratio. Plasma progesterone levels peaked on GD 15.0 in our mice and were already significantly diminished by GD 16.5 signifying the initiation of luteolysis during this period (Cook *et al.*, in press). In rats, the fall in progesterone is associated with an increase in the activity of ovarian 20α-hydroxysteroid dehydrogenase (Bast and Melampy, 1972) leading to the formation of the biologically inactive metabolite of progesterone, 20α-dihydroprogesterone (Wiest, 1970). In addition, progesterone is metabolized by uterine tissues and placenta and this metabolism increases towards term in the rat (Benbow and Waddell, 1995). Stocco and Deis (1996), however, suggest that progesterone withdrawal is also facilitated by a decrease in progesterone synthesis by 3β-HSD which precedes the increase in the 20α-HSD-mediated metabolism of progesterone. On gestational day 19 in rats, exogenous LH leads to a decrease in 3β-HSD, followed by an increase in 20α-HSD and a decrease in serum progesterone, and this effect can be prevented by LH antiserum administration on gestational day 19 (Stocco and Deis, 1996). The effect of LH appears to be direct since LH increases in plasma, and there is a corresponding up-regulation of LH receptor late in gestation (Stocco and Deis, 1996). Luteinizing hormone increases $PGF_{2\alpha}$ and cAMP during luteolysis, and cAMP can then increase the expression of 20α-HSD (Zhong *et al.*, 1998). Luteinizing hormone, $PGF_{2\alpha}$, or fetal/placental removal, hysterectomy are all followed by increases in luteal 20α-HSD activity and decreases in luteal 3β-HSD activity in pregnant rats (Rodway and Kuhn, 1975).

The P450 aromatase was localized to murine granulosa cells and luteal cells in pregnant rats and the estrogen increase with advancing pregnancy in rats may be the result

of an increase in ovarian P450 aromatase activity in mice (Wong *et al.*, 1995). In rats, the late gestation increase in P450 aromatase mRNA (Lephart *et al.*, 1992; Akinola *et al.*, 1997) may be responsible although levels decreased from day 20 until parturition. According to Hickey *et al.* (1988), P450 aromatase increases late in gestation in the corpus luteum of the rat and then decreased at time of parturition. There was little estradiol produced by the late gestation corpus lutea isolated and incubated *in vitro* but corpora lutea from around the time of parturition increased estradiol synthesis despite low enzyme levels. P450 aromatase was unaffected by hCG treatments suggesting that some other factor may be responsible for the parturition-related enhancement in estradiol synthesis. There is also a late gestational increase in androgen (estrogen precursors) (Barkley *et al.*, 1979). Androgen comes from the ovary until it loses its ability to convert progesterone to androstenedione with advancing gestation, at which time the placenta becomes the principal source (Sridaran *et al.*, 1981). Just prior to parturition, estradiol production by the corpora lutea may be supplemented by nonluteal tissues. Alternatively, the estradiol increase may be derived from placental androgens as well as a shunting of ovarian pregnenolone away from progesterone synthesis toward estrogen synthesis. Effectively, there would be an increase in precursor availability for estrogen synthesis.

In rats, cytosolic and nuclear ER are maximal at labour, cytosolic PR increases until term and nuclear PR decrease (Mahesh *et al.*, 1996). Myatt *et al.* (1994) have also reported an increase in nuclear ER and a decrease in PR in rat myometrium. Unfortunately, the generation of mice deficient in ERα or PR were not conducive to further elucidation of the role of these receptors in parturition since infertility plagues both the ERα knockout mouse (Lubahn *et al.*, 1993) and the PR knockout mouse (Lydon *et al.*, 1995).

Effect of Alcohol

Since luteolysis began prior to the administration of ethanol in our mice, ethanol cannot be prematurely initiating luteolysis but may enhance it (Cook *et al.*, in press). The effects of alcohol may be mediated by the regulation of LH action. Alcohol has significant effects on membrane lipids and membrane bound proteins (Hoek and Taraschi, 1988) and may interrupt the signalling cascades triggered by LH binding to its receptor. Ethanol can modify LH action, but the diverse effects of ethanol is heavily dependent upon doses and dosage regimens (acute vs. chronic) which complicates interpretation and correlation of the data. Very little information on acute ethanol effects late in gestation. Studies in non-pregnant animals have shown that acute exposure to alcohol given on proestrous in the rat significantly reduced serum LH levels, estradiol levels and progesterone levels (LaPaglia *et al.*, 1997). In cultured granulosa cells obtained from women undergoing *in vitro* fertilization protocols, a two day exposure to chronic doses of ethanol inhibited LH-induced estradiol and progesterone production (Saxena *et al.*, 1990) and EGF-stimulated progesterone secretion (McKenzie *et al.*, 1995). In JEG choriocarinoma cells (representing placental cells) at concentrations similar to those found in chronic alcoholics, ethanol disrupted basal and cAMP-stimulated estrogen and progesterone secretion in different directions depending on the period of ethanol exposure (Wimalasena, 1994).

Estrogen synthesis throughout pregnancy is important to sustain progesterone production. Thus, alcohol by interfering with LH may reduce this estrogen synthesis leading to a fall in both estrogen and progesterone. Perhaps the estrogen rise observed at term is a compensatory increase in estrogen in attempts to prevent the progesterone decrease thereby leading to a gradual decline in progesterone. Loss of this compensation following alcohol

treatment may lead to an accelerated decline in progesterone levels. This would depend on alcohol having a specific effect on estrogen synthesis. Alternatively, the following scenario can be envisioned. During the first half of pregnancy, LH stimulates androgen synthesis by thecal-interstitial cells which can then be converted to estradiol in the corpora lutea or granulosa cells. During the second half of pregnancy, androgens are derived from placental sources and are responsible for corpora luteal production of estradiol. During parturition, both pathways are active leading to an increase in plasma estradiol levels. Ethanol, by virtue of its inhibition of LH, suppresses one of the two operating mechanisms and abolishes the plasma estradiol increase.

Alcohol may also exert its effects through the modulation of $PGF_{2\alpha}$ action (increasing synthesis, increasing receptor expression) since $PGF_{2\alpha}$ is believed to play a role in luteolysis (discussed in detail later).

Future Studies

It is important to remember that the experiments described herein only examined circulating levels of steroid hormones which may be regulated differently in the tissues. Future experiments into local concentrations is warranted. Furthermore, the levels of the other estrogens (estrone and estriol) as well as estrogen and progesterone metabolites deserves investigation. Steroidogenic enzyme expression and activity should be studied in mice in order to better clarify their roles in term birth and ethanol-induced preterm birth. The direct effect of ethanol on estrogen synthesis should also be examined. Finally, exogenous estrogen can be administered to see whether preterm birth can be instigated.

E. CONCLUSION

In conclusion, both ethanol-induced preterm birth and normal term birth in the mouse were associated with progesterone withdrawal whereas only term birth was characterized by elevated plasma estrogen levels. These results are novel because they show that preterm birth induced by ethanol exposure is different from normal birth at term and suggest that an increase in estrogen may not be essential for parturition to occur. Nevertheless, the plasma estrogen to progesterone ratio is increased at the time of birth in both untreated controls and ethanol-treated mice. This elevation in the estrogen to progesterone ratio supports our hypothesis. Despite this common endpoint, however, it should be noted that different mechanisms were responsible for this alteration in the estrogen to progesterone ratio. In untreated controls, the increase in the estrogen to progesterone ratio at term was a consequence of both a decrease in peripheral progesterone levels and an increase in peripheral estrogen levels. Ethanol-induced preterm birth, on the other hand, exhibited an increase in the estrogen to progesterone ratio that was achieved through progesterone withdrawal alone.

CHAPTER IV. PROSTAGLANDINS

A. INTRODUCTION

Term Birth

There has been surprisingly little profiling of the prostaglandin changes during term parturition in mice. As a consequence, it is necessary to refer to studies conducted in rats. Plasma levels of $PGF_{2\alpha}$ as well as amniotic fluid $PGF_{2\alpha}$ and PGE_2 increased progressively from day 16 until parturition in rats (day 22) (Molnar and Hertelendy, 1990). The chronological changes in tissue prostaglandins late in gestation in the rat were reported by Wilson and Freinkel (1982). They showed that uterine PGF and PGE increased with gestation *in vivo* and *in vitro* whereas no change was detected in placentas. Moreover, the increase was significant by day 19 with further increases during parturition. Later studies by Fang *et al.* (1996) showed that the increase in uterine PGE was an increase in PGE_2, but they did not measure $PGF_{2\alpha}$ levels. Higher levels of PGE_2 and $PGF_{2\alpha}$ were released into culture media *in vitro* when myometrium was obtained from rats about to deliver than from rats on day 15 of their pregnancy (Gu *et al.*, 1990).

Preterm Birth

When gestational length is chemically manipulated, there is an increase in uterine $PGF_{2\alpha}$ and PGE_2 associated with the onset of birth regardless of the chemical used. For example, tamoxifen administration delayed the onset of birth in rats. In these rats, uterine

PGE_2 levels late in gestation were low compared to controls which were about to deliver. However, prior to taxmoxifen-delayed post-term birth, uterine PGE_2 levels did increase to levels which were indistinguishable from term birth (Fang *et al.*, 1996). Similarly, when RU486 was given to induce preterm birth, rat uterine PGE_2 and $PGF_{2\alpha}$ (to a greater extent) increased gradually and remained high until delivery (Fang *et al.*, 1997).

RU486 administration to mice also induced preterm birth and increased PGE_2 and $PGF_{2\alpha}$ production by decidual explants when compared to untreated controls (Dudley *et al.*, 1996). Decidual explants from pregnant mice were able to respond to LPS, IL-1 and TNF by significantly increasing the production of PGE_2 *in vitro* (Dudley *et al.*, 1993). In addition, LPS or IL-1 given *in vivo* to pregnant mice yielded decidual explants which also produced more PGE_2 and $PGF_{2\alpha}$ than controls (Silver *et al.*, 1995; Silver *et al.*, 1997). This increase was attributed to an increase PGHS-2 expression (Silver *et al.*, 1995). In mice, LPS-induced preterm birth was preceded by a rapid induction of PGHS-2 mRNA and protein in uterus, placenta and fetal membranes (Swaisgood *et al.*, 1997). Furthermore, LPS-induced preterm birth could be blocked by the administration of indomethacin, a non-specific inhibitor of prostaglandin synthesis (Kaga *et al.*, 1996). Intrauterine bacterial inoculation (Hirsch *et al.*, 1995) as well as intracervical inoculation of *E. coli* (Reznikov *et al.*, 1999) also resulted in an up-regulation of PGHS-2 and preterm birth in mice. The results of these studies suggested that the gestation-shortening effects of bacteria or bacterial products in mice were mediated, in part, by cytokines and prostaglandins.

Purpose

These studies were conducted to measure the changes in uterine and placental prostaglandin E_2 and $F_{2\alpha}$ with advancing gestation in untreated control mice and mice treated with an acute dose of ethanol. It was hypothesized that PGE_2 and $PGF_{2\alpha}$ would be elevated (1) at the time of term birth relative to levels late in gestation (GD 16.0), and (2) at the time of ethanol-induced preterm birth relative to levels at the appropriate gestational age-matched control (GD 17.75).

B. MATERIALS AND METHODS

1. Materials

Reagents

Prostaglandin $F_{2\alpha}$

Tritiated Tracer: [5,6,8,9,11,12,14,15-^{3}H(N)]-Prostaglandin $F_{2\alpha}$ (^{3}H-$PGF_{2\alpha}$) (New England Nuclear Life Science Products, Boston, MA, Cat. # NET-433)

Standard: Purified $PGF_{2\alpha}$ (Sigma Aldrich, Oakville, ON, Cat. # P-3023)

Antiserum: Rabbit anti-$PGF_{2\alpha}$ (PE Biosystems, Mississauga, ON, Cat. # 8-1002)

Prostaglandin E_2

Tritiated Tracer: [5,6,8,11,12,14,15-^{3}H(N)]-Prostaglandin E_2 (^{3}H-PGE_2) (New England Nuclear Life Science Products, Boston, MA, Cat. # NET-428)

Standard: Purified PGE_2 (Sigma Aldrich, Oakville, ON, Cat. # P-5640)

Antiserum: Rabbit anti-PGE_2 (PE Biosystems, Mississauga, ON, Cat. # 8-1001)

Miscellaneous

Ethyl Acetate (Fisher Scientific, Nepean, Ontario)

100% Ethanol (Commercial Alcohols, Brampton, Ontario)

Formic Acid (Anachemia, Montreal, Quebec)

Scintisafe Plus 50% Scintillation Cocktail (Fisher Scientific, Nepean, Ontario)

Micro BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL)

Solutions and Buffers

Indomethacin Solution	6.25 mM indomethacin (Sigma Aldrich, Oakville, Ontario), 1% sodium bicarbonate, pH 7.3
PVP Buffer	55 mM Tris, 154 mM NaCl, pH 7.4, 0.1% polyvinylpyrrolidone, stored at 4°C
Dextran-Coated Charcoal Solution	0.0625% (w/v) dextran, 0.625% activated charcoal

2. Uterine and Placental Prostaglandin $F_{2\alpha}$

Uterine $PGF_{2\alpha}$ measurements were made in mice from each gestational age in the untreated control time course (n=7-12/time point) and the ethanol-treated time course (n=6-12/time point). In contrast, placental $PGF_{2\alpha}$ levels were determined only at the four key time points (GD 16.0, GD 17.75, Preterm Birth, Term Birth).

Extraction and Protein Assay

$PGF_{2\alpha}$ extraction and measurements were performed according to methods described previously (Cook and Randall, 1997) and modified as follows. Snap frozen uterine or placental tissue was pulverized using liquid nitrogen and a pre-chilled mortar and pestle. Using 12 x 75 mm polypropylene test tubes, 0.05 g of tissue were suspended in 500 µL of indomethacin solution and homogenized for 20 sec on ice with a Brinkman polytron homogenizer (setting 6). Blanks were prepared with buffer alone. To estimate recovery, a tracer solution of ^{3}H-PGE_2 (2,000 cpm/50 µL) was added to the tissue homogenates and blanks before extraction with 1 mL of absolute ethanol. Samples were centrifuged at 850 x *g* for 15 min at 4°C and the pellet was resuspended in distilled water, sonicated for 30 sec and analyzed for protein using a kit for the BCA Protein Assay (Smith, 1985). The supernatant was evaporated under vacuum and samples were re-dissolved overnight in 1 mL phosphate buffered saline before acidification with 95 µL of 2.7 % formic acid (v/v) and extraction into ethyl acetate. After evaporation of the ethyl acetate under vacuum, samples were reconstituted overnight in 500 µL of PVP buffer before quantification by radioimmunoassay. Extraction recovery values were 89.6 ± 1.2 %.

Radioimmunoassay

A standard curve was pipetted using a 1 mg/mL purified $PGF_{2\alpha}$ stock vial and 100 µL aliquots of each standard were prepared and stored at -80°C. On the day of the assay, a standard curve (in triplicate) was thawed. Pre-diluted, frozen and aliquoted quality controls were run in duplicate at the beginning and end of each assay, and samples (50 µL) were diluted with 50 µL of PVP buffer. Total count (T), non-specific binding (NSB) and maximal

binding (Bmax) tubes were also prepared in triplicate with PVP buffer alone. One hundred μL of ^{3}H-$PGF_{2\alpha}$ (10,000 cpm/100 μL) were added to each tube and 100 μL of reconstituted antibody were added to every tube except T and NSB tubes. After the assay was pipetted, tubes were vortexed, covered with parafilm and incubated overnight at 4°C. The next day, 700 μL of ice cold charcoal solution was added to each tube on ice except T tubes which received water. The tubes were vortexed and centrifuged at 850 x *g* for 13 min at 4°C to separate antibody bound and free fractions. Supernatants were then decanted into labeled scintillation vials and 7 mL of scintillation cocktail was added. Vials were shaken well and counted in a Beckman liquid scintillation counter for 1 min. All sample values were expressed as the mean of duplicate measures. Procedural blanks were assayed with the samples and extraction recoveries were determined for all samples and blanks. Sample values were corrected for recovery, blank values were subtracted and values were normalized to protein content. Results were expressed as pg $PGF_{2\alpha}$/mg protein.

Characterization of Assay

The accuracy of the $PGF_{2\alpha}$ assay was determined by measuring known quantities of $PGF_{2\alpha}$ in duplicate in the assay. The results are summarized in Table IV-1. In order to measure the precision of the assay, three separate assays were conducted consisting of T, NSB and Bmax tubes in triplicate, triplicate standard curves and quadruplicate quality controls representing a high pool (750 pg), a middle pool (200 pg) and a low pool (30 pg). The intra- and inter-assay coefficients of variation for each of these quality controls are summarized in Table IV-1. In addition, the sensitivity of the $PGF_{2\alpha}$ assay was 5 pg. With respect to specificity, the antibody for the $PGF_{2\alpha}$ radioimmunoassay has a reported 100%

cross-reactivity with $PGF_{1\alpha}$, but there is no cross-reactivity with other prostaglandins according to the supplier.

Table IV-1. $PGF_{2\alpha}$ Radioimmunoassay Accuracy and Precision.

Mass Added (pg)	Mass Observed (pg)	Intra-Assay Coefficient of Variation (%)	Inter-Assay Coefficient of Variation (%)
750	798.2 ± 20.0	7.0	15.6
200	243.8 ± 13.9	11.7	13.9
30	31.7 ± 1.5	4.1	11.6

n=3-4 per group

Additional Assay Validations

To further confirm the validity of the $PGF_{2\alpha}$ assay, different dilutions (1:1, 1:2) of several samples were run in an assay to ensure that the measured values were proportional to each dilution. In addition, blanks and tissue extracts were prepared using either indomethacin buffer, 1% sodium bicarbonate buffer or phosphate buffered saline. It was determined that the presence of indomethacin in the buffer decreased the $PGF_{2\alpha}$ measurements by half thereby proving its necessity in preventing *in vitro* prostaglandin production. Furthermore, indomethacin did not interfere with the assay since serial dilutions of the samples in the presence of indomethacin yielded results as expected and did not affect the assay.

3. Uterine and Placental Prostaglandin E_2

Uterine PGE_2 levels were measured in mice from the untreated control time course (n=7-13/time point) and the ethanol-treated time course (n=6-12/time point). In contrast, placental PGE_2 levels were determined only at the four key time points (GD 16.0, GD 17.75, Preterm Birth, Term Birth).

Extraction and Protein Assay

PGE_2 extraction and measurements were performed according to methods described previously (Olson *et al.*, 1986) and modified as follows. Snap frozen uterine or placental tissue was pulverized using liquid nitrogen and a pre-chilled mortar and pestle. Using 12 x 75 mm polypropylene test tubes, tissue (0.05 g) was suspended in 1.55 mL absolute ethanol for extraction and homogenized for 20 sec on ice with a Brinkman polytron homogenizer (setting 6). Blanks were prepared with absolute ethanol alone. To estimate recovery, a tracer solution of ^{3}H-PGE_2 (2,000 cpm/50 µL) was added to the tissue homogenates and blanks. Samples were centrifuged at 850 x *g* for 15 min at 4°C and the pellet was resuspended in distilled water, sonicated for 30 sec and assayed for protein using a BCA Protein Assay (Smith, 1985). The supernatant was evaporated under vacuum and samples were reconstituted overnight in 500 µL of PVP buffer before measurement by radioimmunoassay. Extraction recovery values were 88.4 ± 0.6 %.

Radioimmunoassay

A standard curve in triplicate (300 µL each tube) was generated on the day of the assay from a stock vial of 1 mg/mL PGE_2 in 95% ethanol stored at -80°C. Pre-diluted, frozen and aliquoted quality controls were run in duplicate at the beginning and end of each assay. 150 µL of sample was diluted with 150 µL of PVP buffer. Total count (T), non-specific binding (NSB) and maximal binding (Bmax) tubes were also prepared in triplicate with PVP buffer alone. 100 µL of ^{3}H-PGE_2 (10,000 cpm/100 µL) was added to each tube and reconstituted antibody (100 µL) was added to every tube except T and NSB tubes. After the assay was pipetted, tubes were vortexed, covered with parafilm and incubated overnight at 4°C. The next day, 1 mL of ice cold charcoal solution was added to each tube on ice except T tubes which received water. The tubes were vortexed and centrifuged at 850 x *g* for 13 min at 4°C to separate antibody bound and free fractions. Supernatants were decanted into labeled scintillation vials, 7 mL of scintillation cocktail was added and vials were shaken well. Vials were then counted in a Beckman liquid scintillation counter for 1 min. All sample values were expressed as the mean of duplicate measures. Procedural blanks were assayed with the samples and extraction recoveries were determined for all samples and blanks. Sample values were corrected for recovery, blank values were subtracted and values were normalized to protein content. Results were expressed as pg PGE_2/mg protein.

Characterization of Radioimmunoassay

The accuracy of the PGE_2 assay is summarized in Table IV-2 and these values were determined by measuring known quantities of PGE_2 in duplicate in an assay. PGE_2 assay precision was determined as specified for the $PGF_{2\alpha}$ assay except that the value of the low

pool was 25 pg. The intra- and inter-assay coefficients of variation for each of these quality controls are summarized in Table IV-2. The sensitivity of the PGE_2 assay was 5 pg. According to the supplier, the antibody for the PGE_2 radioimmunoassay had a cross-reactivity of 50% with PGE_1, but there was no cross-reactivity with other prostaglandins.

Table IV-2. PGE_2 Radioimmunoassay Accuracy and Precision.

Mass Added (pg)	Mass Observed (pg)	Intra-Assay Coefficient of Variation (%)	Inter-Assay Coefficient of Variation (%)
750	755.8 ± 47.1	8.5	7.0
200	195.9 ± 7.2	3.4	12.6
25	16.9 ± 0.7	5.3	32.8

n=3-4 per group

Additional Extraction and Assay Validations

Initially, the PGE_2 extraction was performed using indomethacin solution and ethyl acetate in accordance with the $PGF_{2\alpha}$ extraction. However, it was determined that the presence of indomethacin or ethyl acetate interfered with the PGE_2 radioimmunoassay resulting in very high PGE_2 values for the extraction blanks. As a consequence, it was necessary to establish an extraction procedure using ethanol. To ensure the validity of this method, blank tubes and tissue tubes were spiked with 0, 400, or 1000 pg of PGE_2 prior to homogenization and then assayed. The results are plotted in Figure IV-1A. Analysis of the blank tubes and tissue tubes revealed that both groups shared similar slopes (blanks = 1.9, samples = 2.3). However, it was noted that the observed values obtained from the assays

were consistently higher than expected (approximately two-fold higher than expected) and this was reflected in the magnitude of the slopes reported above. This was attributed to the degradation of the standard curve aliquots over time and thus, it was decided that fresh standard curves would be pipetted for each assay. Since this test was clearly able to detect relative differences, albeit at double the expected values, it was deemed unnecessary to repeat this test with a freshly pipetted standard curve. Finally, another assay was conducted using extracts obtained from initial tissue masses of 0, 40, 80 and 120 mg and the values of PGE_2 increased accordingly (Figure IV-1B). The results of these tests satisfied our concerns over the ability of the PGE_2 assay to measure relative differences and allowed us to proceed accordingly with confidence.

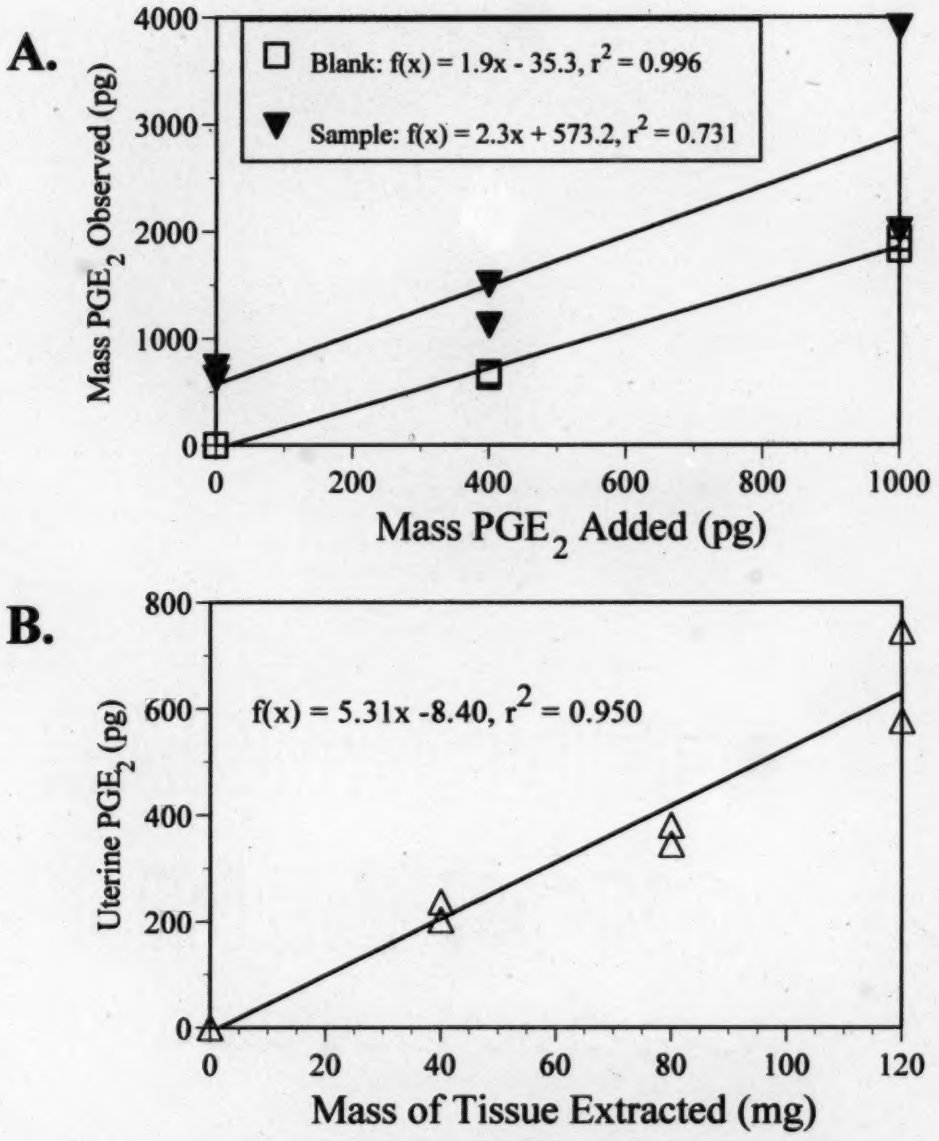

Figure IV-1. Additional PGE_2 extraction and assay validations. (A) The observed values of PGE_2 obtained by RIA were plotted against known concentrations of PGE_2 which were added to samples and blanks prior to assay. (B) Increasing initial tissue masses were extracted and the corresponding PGE_2 levels measured.

4. Statistical Analysis

All results are expressed as the mean ± SEM. In order to detect changes with advancing gestational age, separate one-way ANOVAs (SigmaStat version 2.0, SPSS Inc.) were conducted for the untreated control and ethanol-treated time courses with post-hoc analyses by Tukey's test as required. There was one exception to this rule. When the effect of gestational age on placental prostaglandins in the ethanol-treated group was examined, a Student's t-test was used since only two time points (GD 16.0 and preterm birth) were available. Further analyses with Student's t-tests were utilized to examine differences between the untreated (control) and ethanol-treated (experimental) groups at key time points. These included comparisons between term birth and preterm birth groups as well as comparisons between preterm birth and its gestational age-matched control. A statistical level of significance of $p \leq 0.05$ was chosen.

C. RESULTS

1. Uterine Prostaglandin $F_{2\alpha}$

Gestational age had a significant effect on uterine $PGF_{2\alpha}$ in both the untreated term birth mice ($F(8,72)=4.08$, $p<0.001$) and the ethanol-treated preterm birth mice ($F(7,58)=6.34$, $p<0.001$) as shown in Figure IV-2. In the term birth group, $PGF_{2\alpha}$ levels are low on GD 16.0 (793.5 ± 80.2 pg/mg protein) and gradually increased with advancing gestation until GD 18.5 (2360.2 ± 498.0 pg/mg protein) and the time of term birth (2170.5 ± 186.5 pg/mg protein)

when $PGF_{2\alpha}$ levels were significantly elevated compared to GD 16.0 ($p<0.05$). In the ethanol-treated mice, uterine $PGF_{2\alpha}$ levels also increased with gestational age and were significantly higher on GD 17.5 (1802.2 ± 97.1) and during preterm birth (1609.7 ± 137.7 pg/mg protein) than GD 16.0 ($p<0.05$).

Figure IV-3 depicts the comparison between the term and preterm birth groups. Statistical analysis by student's t-test revealed a significant effect of ethanol on uterine $PGF_{2\alpha}$ levels ($t_{22}=2.42$, $p=0.02$) since there was an increase in term birth $PGF_{2\alpha}$ levels (2170.5 ± 186.5 pg/mg protein) when contrasted to preterm birth levels (1609.7 ± 137.7 pg/mg protein). However, there was no significant difference between uterine $PGF_{2\alpha}$ levels when the preterm birth group was compared to its gestational age-matched control (untreated GD 17.75) ($t_{17}=0.14$, $p=0.89$) even though the preterm birth group is in labour whereas its control is not. This comparison is shown in Figure IV-4.

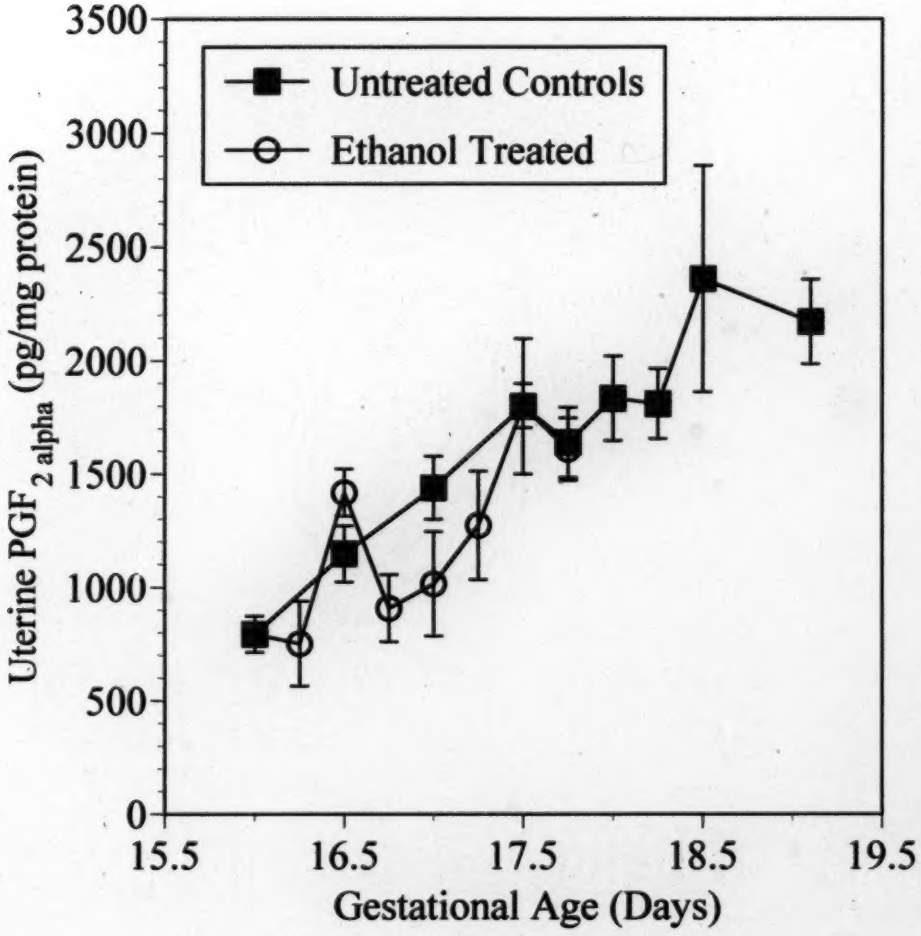

Figure IV-2. The effect of gestational age on uterine $PGF_{2\alpha}$ levels (mean ± SEM) in ethanol-treated (n=6-12 per time point) and untreated controls (n=7-12 per time point). There was a significant effect of gestational age on uterine $PGF_{2\alpha}$ levels in both ethanol-treated mice and untreated controls ($p<0.05$) when compared to GD 16.0.

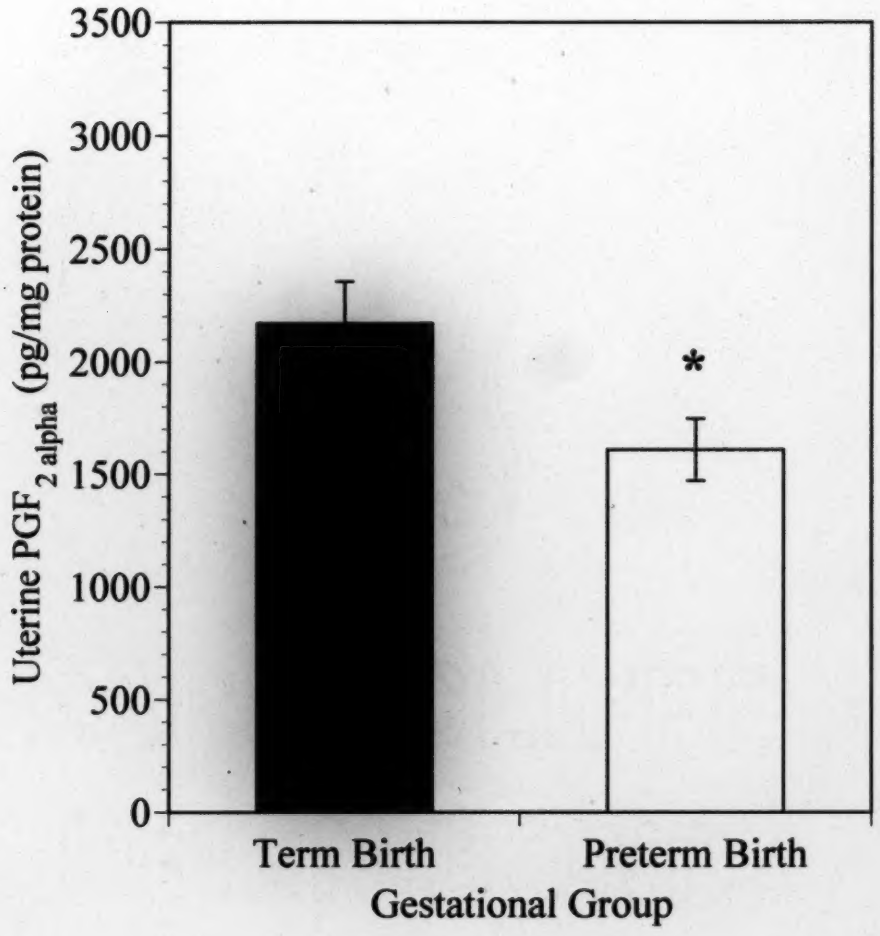

Figure IV-3. The effect of ethanol on uterine $PGF_{2\alpha}$ levels (mean ± SEM) during parturition at term (n=12) and preterm (n=12). The * denotes a significant difference ($p<0.05$).

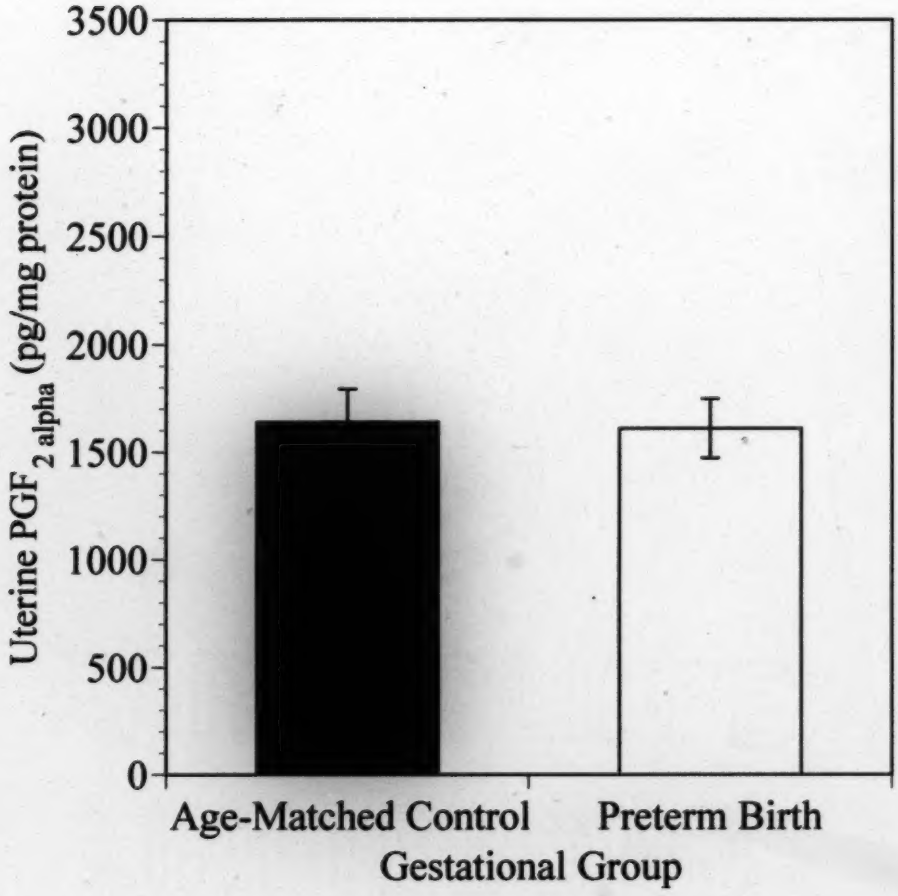

Figure IV-4. The effect of ethanol on uterine $PGF_{2\alpha}$ levels (mean ± SEM) on GD 17.75 in ethanol-treated mice (n=12) and untreated controls (n=7). There was no significant difference ($p>0.05$).

2. Uterine Prostaglandin E_2

Figures IV-5, IV-6 and IV-7 illustrate the uterine PGE_2 time course in ethanol-treated and untreated controls, the comparison between term birth and preterm birth, and the comparison between preterm birth and its gestational age-matched control (untreated GD 17.75), respectively. Levels of uterine PGE_2 were much lower than those observed for uterine $PGF_{2\alpha}$. In the untreated control time course, there was a significant effect of gestation on uterine PGE_2 levels ($F(8,81)=3.511$, $p=0.002$) but post-hoc analysis only revealed a significant difference between GD 18.00 and GD 16.00. In contrast, uterine PGE_2 levels in the ethanol-treated time course were not affected by gestation ($F(7,64)=2.134$, $p=0.054$). Uterine PGE_2 levels in the preterm birth and term birth groups were also not significantly different ($t_{22}=0.36$, $p=0.72$). However, uterine PGE_2 levels during preterm birth (86.6 ± 9.3 pg/mg protein) were significantly elevated over its gestational age-matched control (53.0 ± 7.4 pg/mg protein, $t_{17}=2.49$, $p=0.02$).

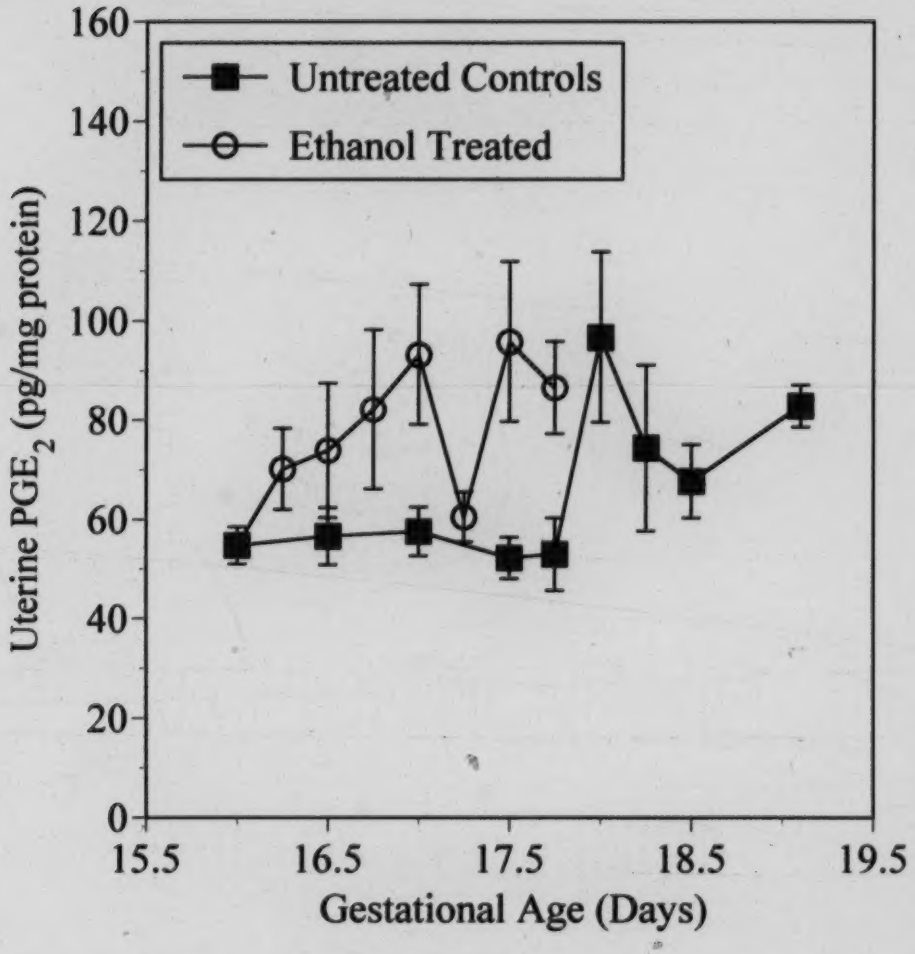

Figure IV-5. The effect of gestational age on uterine PGE_2 levels (mean ± SEM) in ethanol-treated (n=6-12 per time point) and untreated controls (n=7-13 per time point). There was a significant effect of gestational age on uterine PGE2 levels in untreated controls ($p<0.05$) but no effect in ethanol-treated mice ($p>0.05$) when compared to GD 16.0.

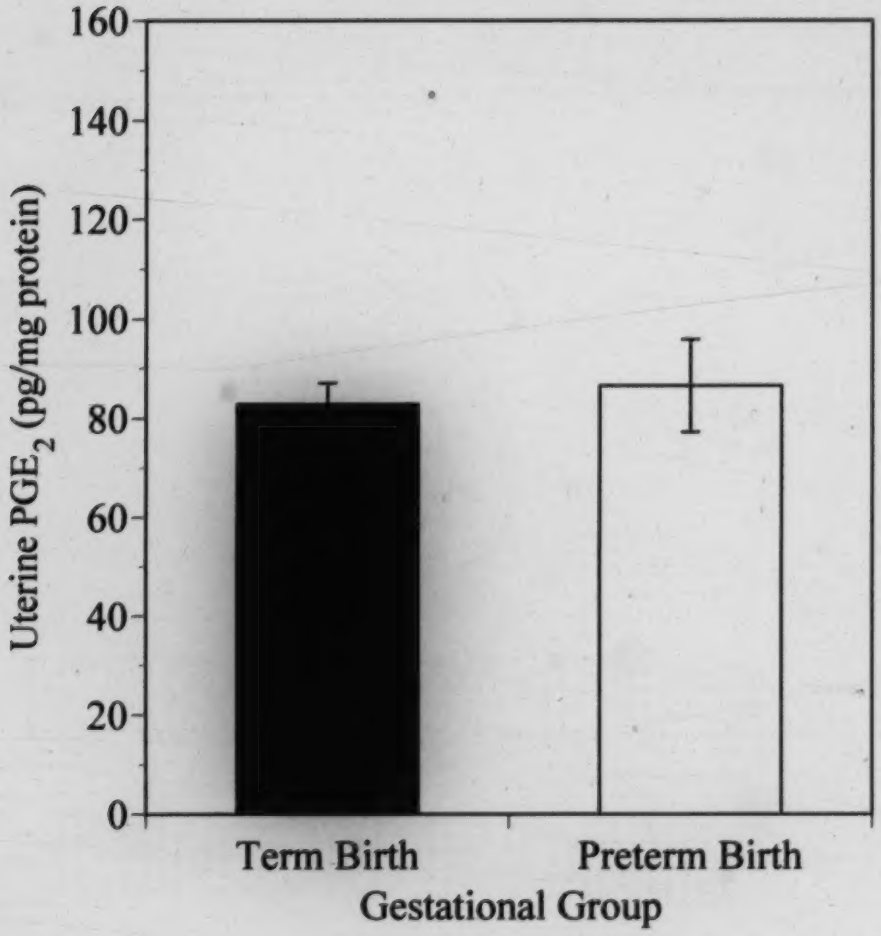

Figure IV-6. The effect of ethanol on uterine PGE_2 levels (mean ± SEM) during parturition at term (n=12) or preterm (n=12). There was no significant difference between the two groups ($p>0.05$).

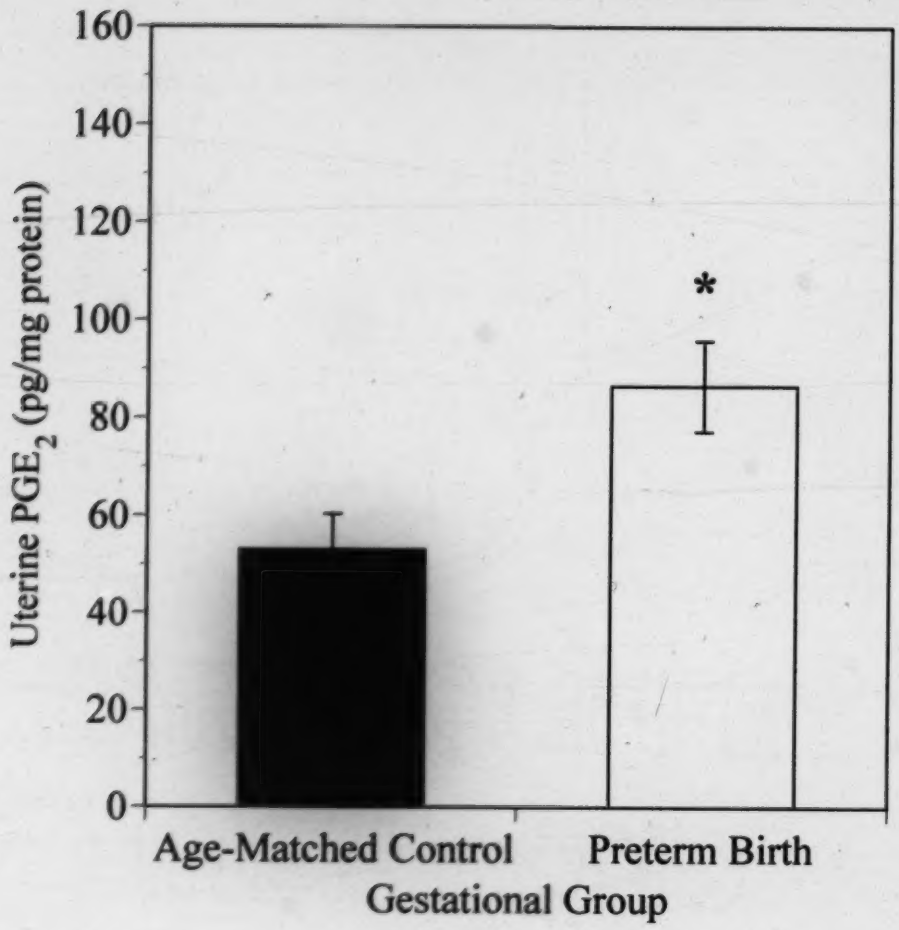

Figure IV-7. The effect of ethanol on uterine PGE_2 levels (mean ± SEM) on GD 17.75 in ethanol-treated mice (n=12) and untreated controls (n=7). The * denotes a significant difference ($p<0.05$).

3. Placental Prostaglandin $F_{2\alpha}$

Preliminary studies (n=6-7 per time point) on placental $PGF_{2\alpha}$ content are depicted in Figure IV-8. There appeared to be a significant effect of gestation on $PGF_{2\alpha}$ levels in untreated controls ($F(2,18)=4.742$, $p=0.024$). Placentas obtained from mice during term birth (1069.3 ± 305.3 pg/mg protein) had $PGF_{2\alpha}$ levels that were significantly elevated above GD 16.00 levels (299.0 ± 50.4 pg/mg protein, $p<0.05$). In ethanol-treated mice, increasing gestational age was also associated with increased placental $PGF_{2\alpha}$ levels ($t_{10}=2.79$, $p=0.019$). However, when the placental $PGF_{2\alpha}$ levels of the preterm birth group (515.2 ± 59.2 pg/mg protein) were compared to term birth (1069.3 ± 305.3) in Figure IV-9 or to its gestational age-matched control (370.8 ± 45.8 pg/mg protein) in Figure IV-10, no significant difference was observed ($t_{11}=1.65$, $p=0.128$ and $t_{10}=1.93$, $p=0.082$, respectively). Placental $PGF_{2\alpha}$ levels were consistently lower than levels observed in the uterus.

4. Placental Prostaglandin E_2

In Figure IV-11, placental levels of PGE_2 measured in a preliminary set of studies (n=6-7 per time point) revealed no effect of gestational age in untreated controls ($F(2,18)=1.046$, $p=0.374$) or ethanol-treated mice ($t_{11}=0.64$, $p=0.53$). Furthermore, there was no significant difference in placental PGE_2 levels between the two birth groups ($t_{12}=0.55$, $p=0.59$) shown in Figure IV-12 or between the preterm birth group and its gestational age-matched control ($t_{11}=0.93$, $p=0.37$) shown in Figure IV-13. Placental PGE_2 levels were comparable to levels measured in the uterus.

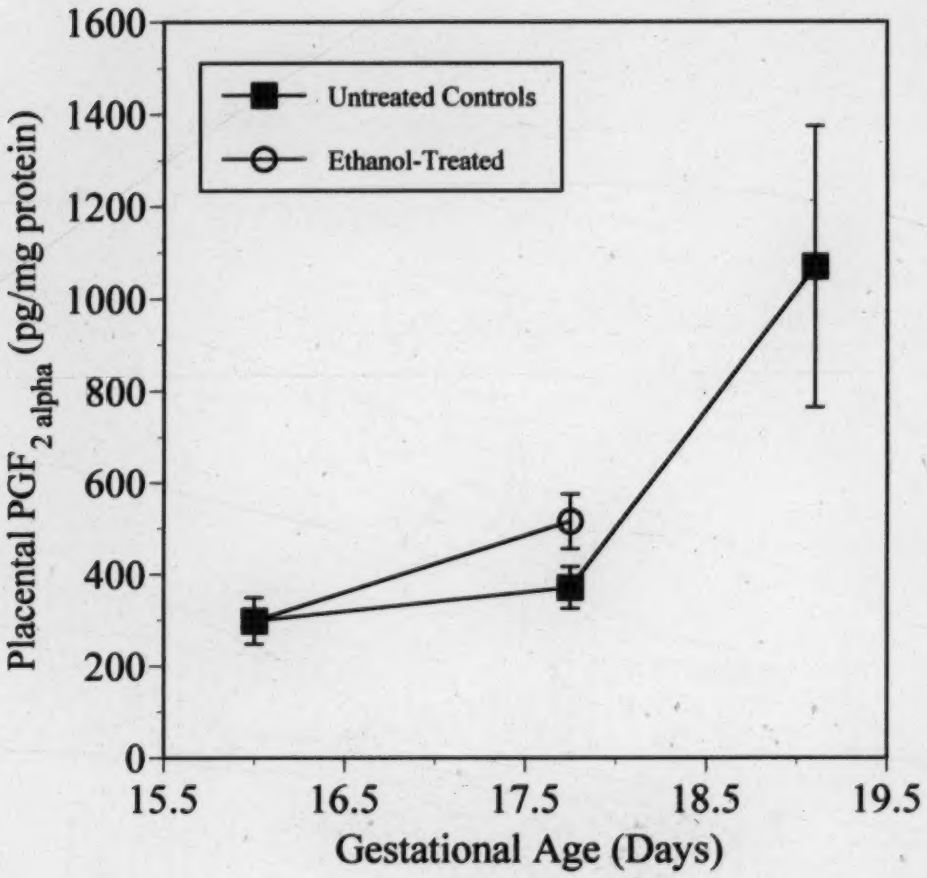

Figure IV-8. The effect of gestational age on placental $PGF_{2\alpha}$ levels (mean ± SEM) in ethanol-treated (n=6 per time point) and untreated controls (n=6-7 per time point). There was a gestational age-related difference when compared to GD 16.0 in untreated controls ($p<0.05$) and ethanol-treated mice ($p<0.05$).

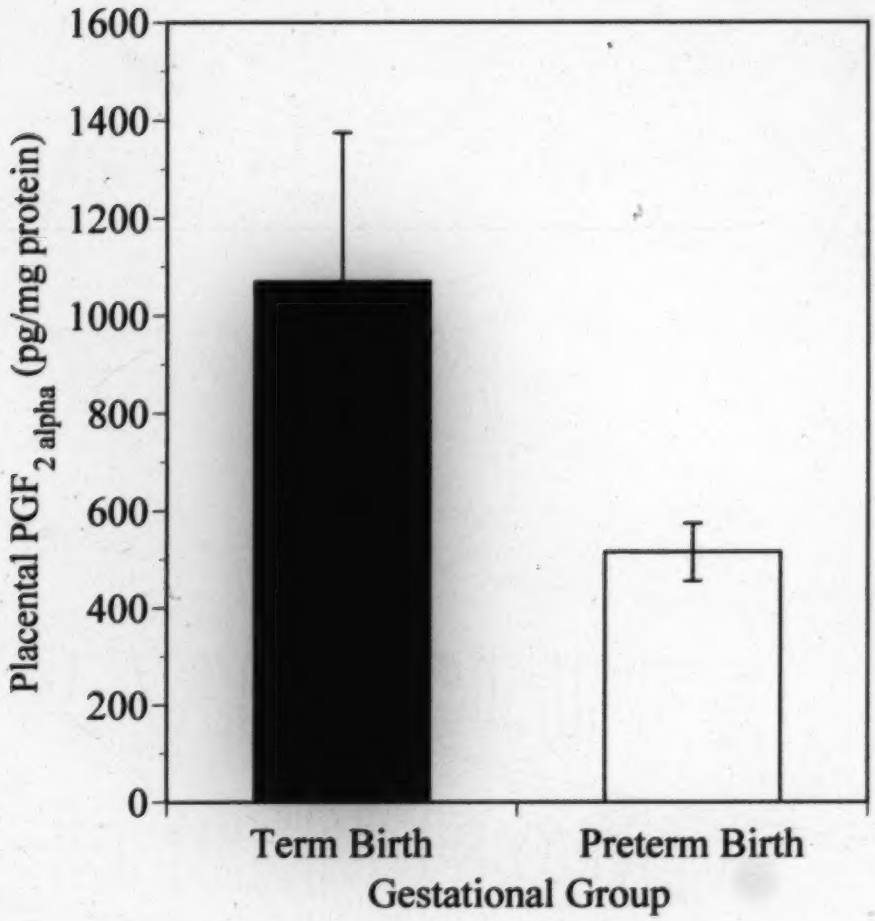

Figure IV-9. The effect of ethanol on placental $PGF_{2\alpha}$ levels (mean ± SEM) during parturition at term (n=7) and preterm (n=6). There is no significant difference between the two groups ($p>0.05$).

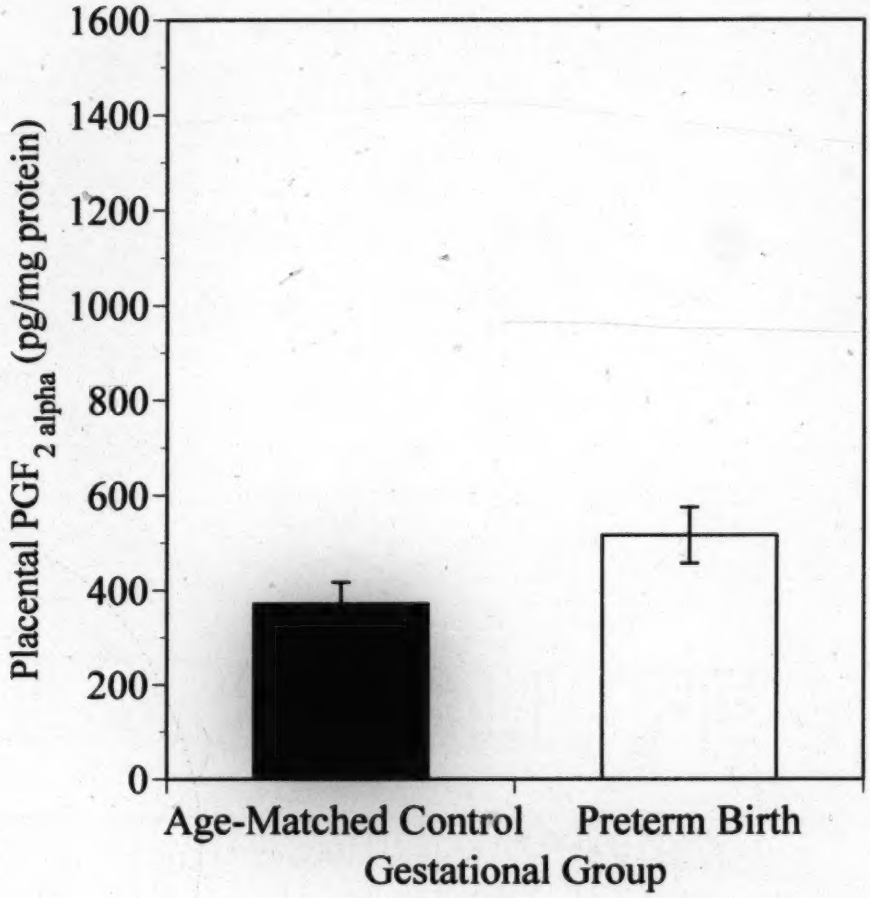

Figure IV-10. The effect of ethanol on placental $PGF_{2\alpha}$ levels (mean ± SEM) on GD 17.75 in ethanol-treated mice (n=6) or untreated controls (n=6). There was no effect of ethanol ($p>0.05$).

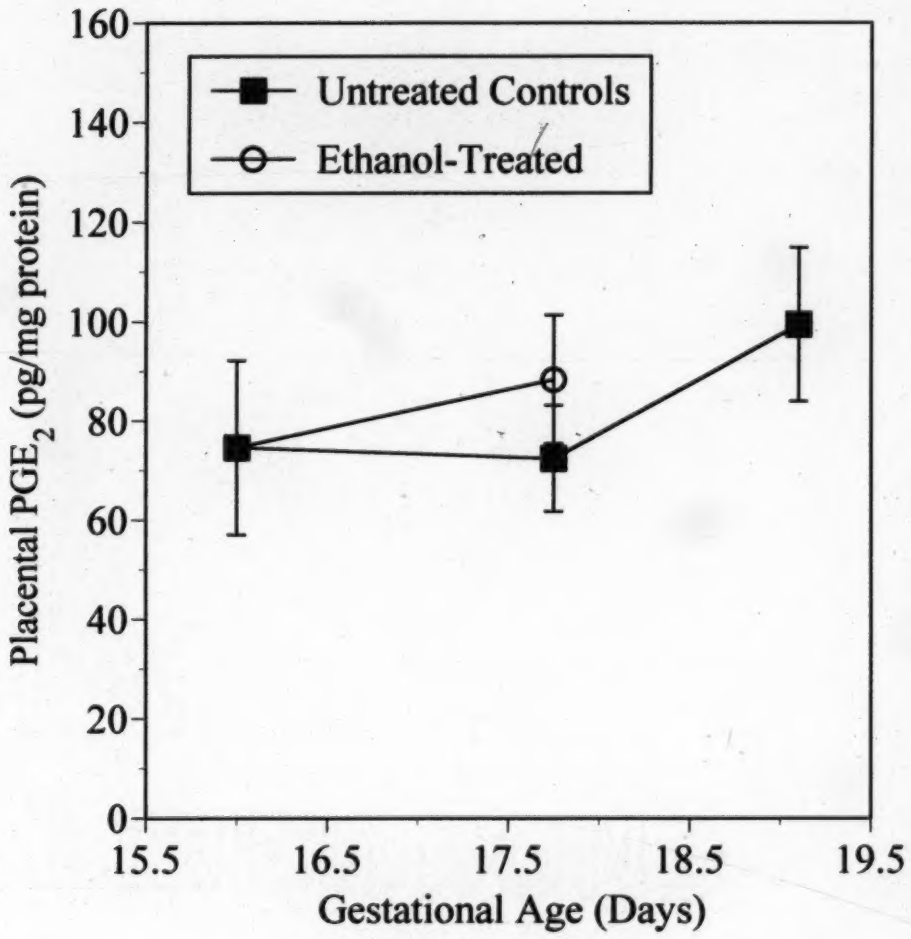

Figure IV-11. The effect of gestational age on placental PGE_2 levels (mean ± SEM) in ethanol-treated (n=6-7 per time point) and untreated controls (n=6-7 per time point). There was no gestational age-related difference in either group when compared to GD 16.0 ($p>0.05$).

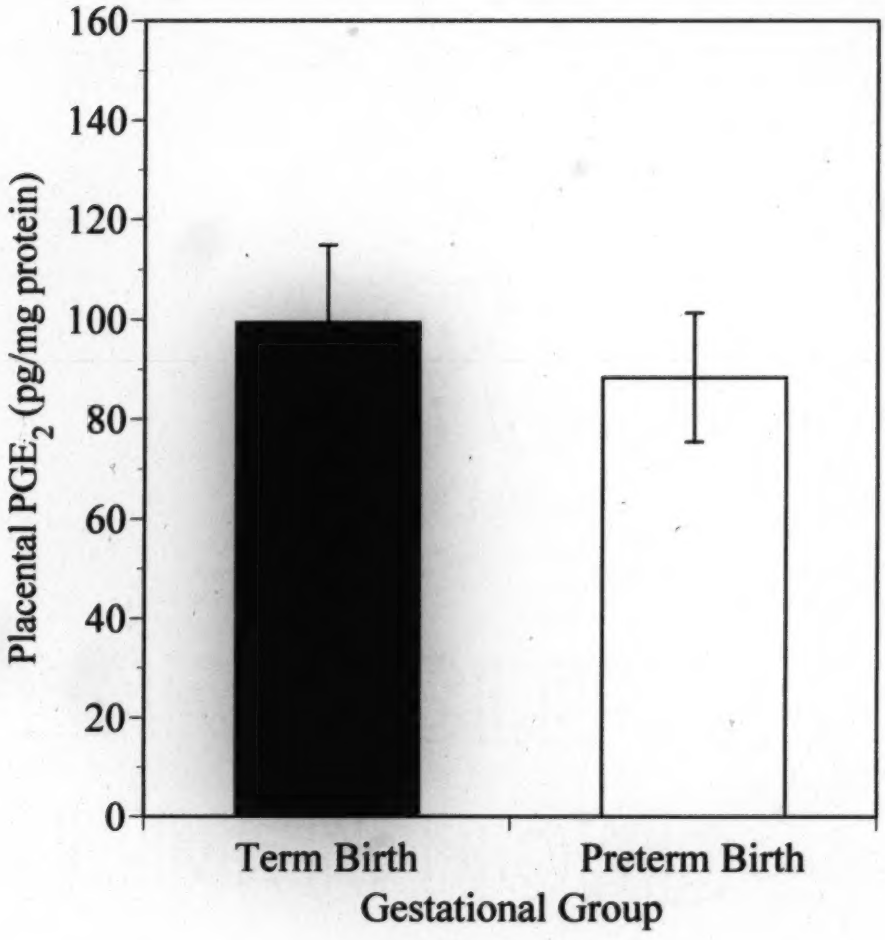

Figure IV-12. The effect of ethanol on placental PGE_2 levels (mean ± SEM) during parturition at term (n=7) or preterm (n=7). There was no significant difference between the two groups ($p>0.05$).

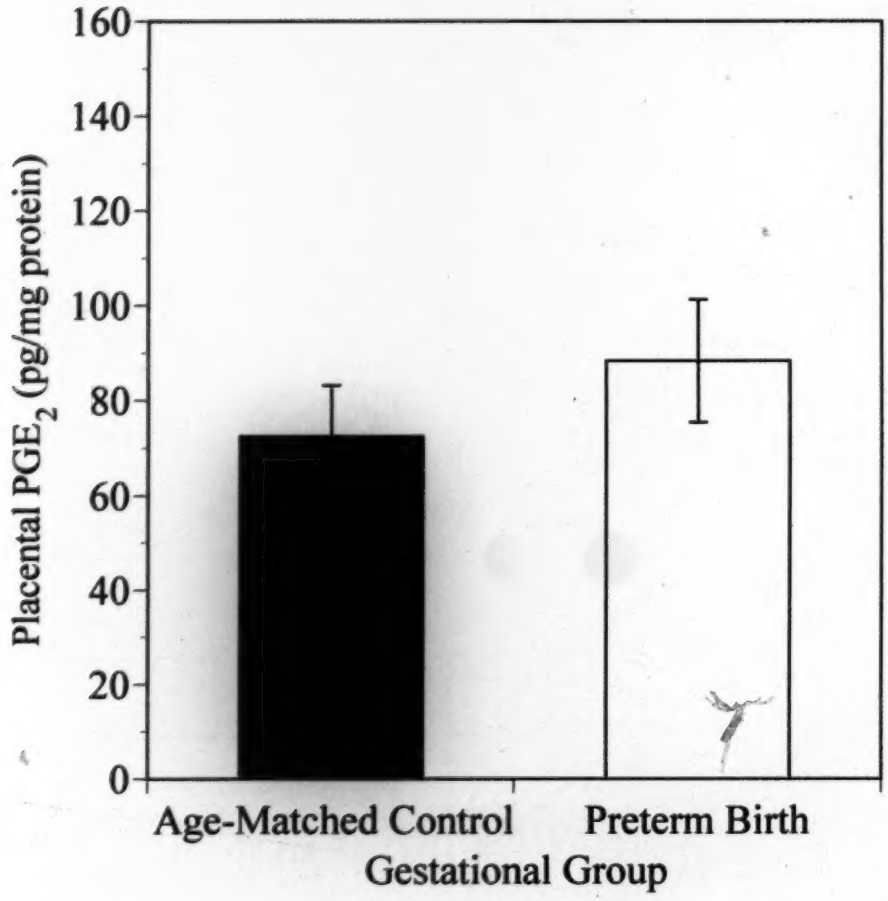

Figure IV-13. The effect of ethanol on placental PGE_2 levels (mean ± SEM) on GD 17.75 in ethanol-treated mice (n=7) or untreated controls (n=6). There was no significant effect of ethanol ($p>0.05$).

D. DISCUSSION

The results of these experiments indicate that uterine PGE_2 and $PGF_{2\alpha}$ were elevated prior to birth, either at term and preterm when compared to GD 16.0. However, whereas uterine PGE_2 was elevated over its gestational age-matched control at the time of preterm birth, there was no difference in uterine or placental $PGF_{2\alpha}$ levels between these two groups despite the fact that the ethanol-exposed group was in labour and had delivered one pup.

At term, the source of prostaglandin increase is believed to be an increase in the synthesis of prostaglandins by PGHS. However, profiles of the two isoforms of PGHS in rodents have revealed discrepancies in their patterns of expression. Dong *et al.* (1996) report an increase in both PGHS-1 and -2 mRNA and protein in pregnant rat uteri during gestation with further increases with parturition. Myatt *et al.* (1994) showed an increase with gestation in PGHS-1 in rat myometrium but no PGHS-2 was detectable. Studies in our lab (Cook *et al.*, manuscript in preparation) have shown an increase in the mRNA relative abundance for PGHS-2 in association with term and preterm birth. Naproxen (prostaglandin synthesis inhibitor) could prolong gestation in rats (Chan *et al.*, 1988).

When gene knockouts are not lethal, they can be useful in studying function, but it is interesting that different phenotypes can be observed through the disruption of the same gene in different genetic backgrounds (William and Dubois, 1996). Uozumi *et al.* (1997) and Bonventre *et al.* (1997) generated $cPLA_2$ knockout mice which had smaller litters and failed to undergo labour at term but did deliver live, non-viable offspring post-term. Induction of labour at term with RU486 rescued the pups. These data suggest that $cPLA_2$ liberates arachidonic acid for PGHS to convert ultimately to $PGF_{2\alpha}$ which causes luteolysis and

induction of labour. The mating of male and female mice deficient in PGHS-1 also produced less viable offspring (Langenbach *et al.*, 1995). Unfortunately, PGHS-2 knockout mice (Dinchuk *et al.*, 1995; Morham *et al.*, 1995; Lim *et al.*, 1997) suffered from reduced viability (nephropathy) and infertility due to ovulatory failure. The anovulation in PGHS-2 knockouts could be restored by concurrent treatment with gonadotropin stimulation and PGE_2 or IL-1β (Davis *et al.*, 1999).

During preterm birth, the increase in uterine PGE_2 levels over gestational age matched controls may cause sufficient uterine contractility to result in the preterm expulsion of pups. Binding of PGE_2 with its receptors either leads to contraction (EP_1, EP_3) or relaxation (EP_2, EP_4). In rats, EP_2 mRNA decrease from day 16 until parturition (Brodt-Eppley *et al.*, 1998). However, PGE_2 is conventionally thought to play a more important role in cervical ripening conceding contractile effects to the more potent $PGF_{2\alpha}$. In the ethanol-treated preterm birth group, although $PGF_{2\alpha}$ levels were elevated relative to GD 16.0, they were not different from levels observed in gestational age-matched controls. Nevertheless, the preterm birth group was in labour whereas the untreated control was not. This discrepancy suggests that the bioactivity of $PGF_{2\alpha}$ may be enhanced through other mechanisms other than increased synthesis. For example, $PGF_{2\alpha}$ bioactivity may be increased through the up-regulation of receptor expression or the down-regulation of local metabolism. Accordingly, studies in our lab have shown an increase in FP receptor mRNA associated with both birth at term and preterm birth (Cook *et al.*, manuscript in preparation). We do not have direct binding data, however. There was an increase in myometrial $PGF_{2\alpha}$ and PGE_2 binding sites around parturition in rats (Molnar and Hertelendy, 1990) although the authors did not distinguish between the EP subtypes. Brodt-Eppley *et al.* (1998) reported that FP

increased from late in gestation until delivery in pregnant rats. It is also possible that the level of uterine $PGF_{2\alpha}$ present on GD 17.75 in the untreated controls was adequate to elicit contractions but the uterus was unable to respond due to low receptor levels. This would imply that the levels observed during term birth were in excess.

Prostaglandin Actions

In pregnancy, prostaglandins are believed to be important mediators of uterine contractility as well luteolysis. While uterine and placental prostaglandins may exert their effects by directly eliciting uterine contractions, they are also poised to play a role in promoting the regression of the corpus luteum (luteolysis). The contractile effects of prostaglandins can be enlisted at any stage in gestation. During early gestation, $PGF_{2\alpha}$ is an efficient abortifacient and during late gestation, PGE_2 and $PGF_{2\alpha}$ are used clinically for the induction of labour.

In rats, $PGF_{2\alpha}$ given on day 18 can induce preterm birth on day 20 (Alexandrova and Soloff, 1980). $PGF_{2\alpha}$ treatment is associated with an increase in myometrial ER and oxytocin receptor, both of which are abolished by co-administration of progesterone. It was suggested that the preterm birth induced by $PGF_{2\alpha}$ was the result of its luteolytic action rather than its contractile effects because progesterone withdrawal leads to the same receptor changes.

Created by homologous recombination, mice deficient in the FP receptor were fertile and able to establish and maintain pregnancy but were unable to deliver their fetuses at term (Sugimoto *et al.*, 1997). In these mice, the expected withdrawal of progesterone in serum did not occur and ovariectomy was able to reverse this effect and allowed delivery to occur. From these data, it was suggested that the interaction of FP with $PGF_{2\alpha}$ in ovarian luteal cells

was essential to induce luteolysis. In 1998, Gross *et al.* reported that the maternal disruption of the PGHS-1 gene in mice significantly delayed parturition. Impaired luteolysis was again indicated by elevated serum progesterone levels at term. In our studies, the late gestation increase in uterine $PGF_{2\alpha}$ may be involved in the progression of luteolysis. Thus, the additional increase observed with term birth may be important for uterine contractility. In ethanol-treated mice, the elevation in uterine FP mRNA during preterm birth (Cook *et al.*, manuscript in preparation) would suggest a partial diversion of the elevated $PGF_{2\alpha}$ levels towards contractile activities. Wang *et al.* (1993) showed that the uterus was not the sole source of luteolytic $PGF_{2\alpha}$ in pseudopregnant rats since a decrease in progesterone still occurred, albeit later, in hysterectomized rats. A fall in serum progesterone levels is associated with LPS-induced preterm birth in mice (Fidel *et al.*, 1998). Since LPS is a potent stimulator of prostaglandin production, it is tempting to speculate whether these prostaglandins cause luteolysis thereby decreasing peripheral steroid levels.

Effect of Alcohol

Ethanol may indirectly enhance luteolysis by influencing levels of prostaglandin bioactivity. It has been demonstrated that ethanol exposure in rodents can alter prostaglandin levels. Chronic ethanol feeding in rats increased the uterine production of PGE_2 and $PGF_{2\alpha}$ in vitro (Franchi *et al.*, 1988) and prostaglandin metabolism via PGDH. An acute, single injection of ethanol also elevates uterine prostaglandin production *in vitro* in rats (Jaerbaum *et al.*, 1992). Ethanol disrupts membrane structure to enhance membrane fluidity (Goldstein *et al.*, 1986), membrane lipids and membrane-bound proteins (Hoek and Taraschi, 1988). Ethanol increases fluidity of membrane by inducing activation of PLA_2 (Salem and Karanian,

1988). Furthermore, ethanol-induced preterm birth can be abrogated through the inhibition of PGHS, either in a non-isoform specific manner (aspirin) (Cook and Randall, 1997) or in an isoform-specific manner (nimesulide for inhibition of PGHS-2 and valerylsalicylate for inhibition of PGHS-1) (Cook, 1997). Evidence of increased prostaglandin bioactivity after alcohol consumption is provided by Pennington *et al.*,1981). Following chronic alcohol treatment in rats, there was a decrease in maternal kidney PGDH activity and amnion PGDH at term. Ethanol increases PGE in combined uterine/embryo tissue of mice and this blocked by aspirin (Anton *et al.*, 1990).

Future Studies

A profile of changes in prostaglandin levels is needed in other compartments tissues such as the ovaries, amniotic fluid and fetal membranes. Changes in PLA_2, EP subtypes and prostaglandin metabolism (PGDH) and metabolites should also be investigated in this mouse model.

E. CONCLUSION

The data presented herein are novel because they profile the changes in uterine and placental prostaglandin levels in the mouse during late gestation and parturition at term. In addition, this is the first time that the effect of acute ethanol exposure on uterine prostaglandins has been studied in the context of preterm birth. The results of this study refute our hypothesis. Although uterine $PGF_{2\alpha}$ levels increased with increasing gestational age in both the untreated controls and the ethanol-treated mice, uterine $PGF_{2\alpha}$ levels in the

ethanol-treated mice were not elevated relative to gestational age-matched controls at the time of preterm birth. In contrast, uterine PGE_2 levels were elevated at the time of term birth and preterm birth. However, the lower levels of PGE_2 relative to $PGF_{2\alpha}$ and the smaller magnitude of an increase casts doubt on the biological importance of this increase. Thus, the profile of $PGF_{2\alpha}$ and PGE_2 differs between term and ethanol-induced preterm birth.

CHAPTER V. CYTOKINES

A. INTRODUCTION

There is evidence suggesting a role for TH1, TH2, pro-inflammatory and anti-inflammatory cytokines during pregnancy in mice, but a role during parturition at term has not been substantiated. In contrast, the involvement of pro-inflammatory and anti-inflammatory cytokines in infection-related preterm labour is undisputed. Whether these cytokines influence the pathogenesis of other aetiologies of preterm birth remains unknown. Many spontaneous abortions which are independent of genetic abnormalities are immunologically mediated and if these abortions are considered extreme instances of preterm birth, then an infection-independent role for cytokines can be postulated.

Term Birth

A role for TH1/TH2, pro-/anti-inflammatory cytokines during term birth in mice is inferred from evidence accumulated in support of a role for cytokines during pregnancy. Similar to humans, mice tended to favour antibody production against foreign antigens over cell-mediated immunity (Dresser, 1991) during pregnancy. Moreover, normally resistant C57BL/6 mice had an increased severity of *Leishmania major* infection during pregnancy because of a bias away from the curative cell-mediated immune response (Krishnan, *et al.*, 1996a). The preference for these immune responses during pregnancy can be translated into preferences for the TH2 and anti-inflammatory cytokine profiles over the TH1 and pro-inflammatory cytokine profiles. Thus, parturition may represent a shift in the cytokine

balance away from TH2 and anti-inflammatory cytokine synthesis towards TH1 and pro-inflammatory cytokines.

In mice, low levels of IFN–γ, a TH1 cytokine, in placental/decidual single cell cultures were detected only in the first trimester. Towards the end of gestation, Delassus *et al.* (1994) showed that there was an increase in IFN-γ mRNA. In contrast to studies which suggested that IFN–γ is incompatible with pregnancy (Raghupathy, 1997), Ashkar and Croy (1999) demonstrated that IFN–γ produced by mid-gestational uterine NK cells contributed to the normal health of the decidua in mice. Normal pregnancies were also characterized by the spontaneous release of IL-4 and IL-10, TH2 and anti-inflammatory cytokines in placental/decidual single cell cultures and peripheral blood cells throughout pregnancy (Lin *et al.*, 1993; Dudley *et al.*, 1993 and Delassus *et al.*, 1994). There was some evidence that placental IL-10 mRNA decreased towards term (Delassus *et al.*, 1994). However, the administration of IL-10 antibodies to mice did not affect the duration of gestation (Rijhsinghani *et al.*, 1997). According to De *et al.* (1992), uterine and placental IL-1 and IL-6 (pro-inflammatory cytokines) decreased whereas a uterine TNF (pro-inflammatory cytokine) peak was detected before parturition. TNF was absent in the placenta. However, further study is required on changes in the balance of these cytokines late in gestation and at the time of normal term parturition.

Preterm Birth

Most of the research on cytokines in parturition was conducted in the context of infection-associated preterm birth. Consequently, the models of preterm birth in the mouse focussed on bacteria and bacterial products (*i.e.* lipopolysaccharide or endotoxin) as stimuli

which engendered immune responses such as inflammation (immune recruitment and cytokine production). Based upon evidence from human studies relating decidual interleukin-1 (IL-1) levels to infection-associated preterm birth, it was established that interleukin-1 treatment could induce preterm birth in mice (Romero *et al.*, 1991b). With the discovery that decidual explants from untreated mice were responsive to bacterial endotoxin (LPS), IL-1β and TNF-α (Dudley *et al.*, 1993), additional models were developed using endotoxin or bacteria themselves as the trigger. The route of administration of bacteria or endotoxin could be intraperitoneal (Fidel *et al.*, 1994; Kaga *et al.*, 1996), intrauterine (Hirsch *et al.*, 1995) or intracervical (Reznikov *et al.*, 1999). In each instance, preterm birth was induced following treatment and was associated with time-dependent elevations in TNF-α, IL-1 and IL-6 in serum and amniotic fluid (Fidel *et al.*, 1994), as well as uterus (Hirsch *et al.*, 1995; Reznikov *et al.*, 1999). More specifically, uterine and placental IL-1α, IL-1β and IL-6 increased over time after *E. coli* infection in pregnant mice (Hirsch *et al.*, 1999) whereas TNF-α and the anti-inflammatory interleukin-1 receptor antagonist (IL-1ra) increased in a time-dependant fashion only in uterus. The elevated production of these pro-inflammatory cytokines as well as a lack of a corresponding increase in placental IL-1ra suggested a shift away from anti-inflammatory cytokine dominance towards pro-inflammatory dominance prior to infection-associated preterm birth. However, endotoxin induced preterm birth was not prevented by blocking IL-1 or TNF activity using IL-1ra or soluble TNF Receptor fusion protein (Fidel *et al.*, 1997). In an infection-independent model, decidual explants obtained from mice in RU486-induced preterm labour also produced higher levels of IL-6 than controls (Dudley *et al.*, 1996). Based on the evidence presented above, pro-inflammatory and anti-inflammatory

cytokines are important mediators of LPS-induced preterm birth, but their contribution to other methods of inducing preterm labour requires further elucidation.

Abortions

In pathophysiological murine pregnancies such as the resorption-prone mating combination CBA x DBA/2, placental production of IL-4 and IL-10 is deficient and resorptions are accompanied by increases in TNF–α and IFN–γ (Chaouat *et al.*, 1990). Alloimmunization enhances placental IL-4 and IL-10 and decreases resorption. Finally, administration of recombinant IL-10 decreases the resorption rate (partially decreased by anti-IFN-γ or anti-TNF antibody) but administration of anti-IL-10 antibody increased the incidence of resorption (Chaouat *et al.*, 1995). Low doses of anti-TNF antibody reduced resorption rates (Chaouat *et al.*, 1990). *Leishmania* infection in pregnant C57BL/6 mice resulted in increased resorption rates, decreased placental IL-4 and IL-10 and increased placental IFN-γ and TNF (Krishnan *et al.*, 1996b). Administration of TNF-α or IFN-γ caused increased fetal resorptions (Haimovici *et al.*, 1991) and terminated pregnancy (Chaouat *et al.*, 1990; Parant, 1990). Stress-triggered abortion in mice could be blocked by soluble receptors which neutralized TNF–α (Arck *et al.*, 1997) and soluble TNF receptors have been detected in maternal plasma (Kupferminc *et al.*, 1995). Finally, there is an increase in TNF–α mRNA and protein in the placentas of mice with induced (cyclophosphamide-treated) or spontaneous pregnancy loss (Gorivodsky *et al.*, 1998). It should be noted that TNF-α is normally expressed in the female reproductive tract and may play a role in normal reproductive function (reviewed in Terranova *et al.*, 1995). Resorption sites are infiltrated by NK cells (Gendron and Baines, 1988) and anti-NK antibodies can prevent abortions

(DeFougerolles and Baines, 1987; Baines *et al.*, 1997). Lipopolysaccharide-induced abortion was mediated by IL-1 (Silver *et al.*, 1997) and TNF (Gendron *et al.*, 1990; Silver *et al.*, 1994). Hence, the TH1/TH2 and anti-inflammatory/pro-inflammatory cytokine subsets play pivotal roles in murine abortions.

Purpose

There is a paucity of data with respect to the involvement of TH1, TH2, pro-inflammatory and anti-inflammatory cytokines during parturition at term in mice although their importance in pregnancy maintenance has been delineated in some detail. Furthermore, the effect of ethanol on these cytokines subsets has not been examined in the context of preterm birth. It was hypothesized that there would be a shift away from TH2 and anti-inflammatory cytokines towards TH1 and pro-inflammatory cytokine production preceding ethanol-induced preterm birth and normal term birth. In order to address this hypothesis, IFN–γ was chosen as a representative of the TH1 subset, IL-10 was selected as a representative of both TH2 and anti-inflammatory cytokines and TNF was designated the pro-inflammatory cytokine representative. These studies were conducted to profile the chronological changes in placental and amniotic fluid IFN–γ, IL-10, and TNF during term birth and ethanol-induced preterm birth. By assessing the changes in these specific cytokines, it will be possible to determine whether there is a shift in the TH1 and TH2 ratio or the pro-inflammatory and anti-inflammatory balance to favour immune responses which terminate pregnancy at the time of birth.

B. MATERIALS AND METHODS

1. Materials

Reagent Stocks, Antibodies, Standards, Conjugates

Extraction

Leupeptin (Sigma Aldrich, Oakville, Ontario, L-2884, 10 mg/mL in H_2O)

Aprotinin (Sigma Aldrich, Oakville, Ontario, A-1153, 100 U/mL in H_2O)

Phenylmethylsulfonyl Fluoride (PMSF) (ICN, 800263, 100 mM in DMSO)

Normal Goat Serum (Sigma Aldrich, Oakville, Ontario, S-6898)

ELISA

All antibodies and standards were provided courtesy of the Timothy R. Mosmann laboratory (University of Alberta). The antibodies and recombinant standards were produced by PharMingen Canada (Mississauga, Ontario) and the Mosmann laboratory, respectively.

Purified capture antibody	IL-10: (clone: JES5-2A5, rat IgG1, Cat. # 18141D)
	IFN–γ: (clone: R4-6A2, rat IgG1, Cat. # 18181D)
	Isotype Control: (clone: R3-34, IgG1, Cat. # 11011D)
Biotinylated detection antibody	IL-10: (clone: SXC-1, rat IgM, Cat. # 18152D)
	IFN–γ: (clone: XMG1.2, rat IgG1, Cat # 18112D)
Recombinant mouse	IL-10: (Li *et al.*, 1994)
	IFN–γ: (Sad *et al.*, 1995)

Streptavidin / Horseradish Peroxidase Conjugate (Jackson Immunochemicals, 016-030-084)

Defined and Filtered Fetal Bovine Serum (HyClone Cat. No. SH30070.03)

30% H_2O_2 (Fisher Scientific, Nepean, Ontario)

Skim Milk Powder (Carnation)

TMB (3,31,5,51-tetramethylbenzidine) (Sigma Aldrich, 10 mg/mL in DMSO)

Bioassay

The L929-8 cell line, antibodies, recombinant standards and media were generous gifts of the Larry J. Guilbert laboratory (University of Alberta).

Rabbit Anti-Mouse TNF Antibody (Guilbert laboratory, University of Alberta)

Mouse Recombinant TNF from E. Coli (Genentech, San Francisco, CA)

Actinomycin D (Boehringer Mannheim, Laval, Quebec)

Iscove's Modified Dulbecco's Media (IMDM) (Gibco BRL, Burlington, Ontario)

Qualified Fetal Bovine Serum (FBS) (Gibco BRL, Burlington, Ontario, 26140-079)

Heat Inactivated Fetal Bovine Serum (FBS) (Gibco BRL, Burlington, Ontario)

Trypsin-EDTA Solution (0.05% trypsin, 0.53 mM EDTA) (Gibco BRL, 25300-054)

Solutions and Buffers

Extraction

Extraction Buffer Stock 0.1 M Tris, 0.25 M Sucrose, 1 mM EDTA in milli-Q H_2O, pH 6.8, stored at 4°C

Extraction Buffer	5% (v/v) normal goat serum, 1mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide, 5 µg/mL Leupeptin, and 0.15 U/mL Aprotinin in Extraction Buffer Stock, prepared just prior to use

ELISA

PBS	1.9 mM $NaH_2PO_4 H_20$, 8.6 mM K_2HPO_4, 136.9 mM NaCl in milli-Q H_2O, pH 7.3, stored at room temperature
PBST	0.05% (v/v) Tween 20 in PBS, stored at room temperature
2nd Antibody Buffer Stock	0.05% (v/v) Tween 20, 1% (w/v) bovine serum albumin in PBS, 0.2 µm filtered, stored at 4°C
2nd Antibody Buffer	5% (w/v) skim milk powder, 5% normal goat serum in 2nd Antibody Buffer Stock
TMB Substrate Solution	100 µg/mL TMB, 50 mM sodium acetate pH 5.5, 0.003% (v/v) H_2O_2 in milli-Q H_2O

Bioassay

Growth Media	10% Qualified FBS in IMDM, stored at 4°C
TNF Assay Media	5% (v/v) heat inactivated FBS in IMDM, stored at 4°C
Neutral Red Solution	0.05% (w/v) neutral red in saline, filtered, stored in dark at 4°C
NaH_2PO_4 Alcohol Solution	0.05 M NaH_2PO_4 in 50% (v/v) ethanol, stored at room temperature

2. Placental Single Cell Suspension Culture

At the time of tissue collection, three placentas were randomly chosen from each female, and pooled into a petri dish containing 10 mL of R8 Buffer (8% (v/v) heat inactivated FBS, 0.1% (v/v) 2-mercaptoethanol, 40 µg/mL gentamycin in RPMI 1640, Gibco BRL). A single cell suspension was obtained by dispersing the placental cells between two frosted glass slides. Aggregates were allowed to settle, the placental cell suspension was centrifuged at 850 x *g* for 5 min at room temperature. The supernatant was decanted and the pellet was re-suspended in R8 at a concentration of $1x10^6$ cells/mL. Cells were incubated in 24-well plates for 2 h at 37°C in a humidified 5% CO_2 incubator. Following the incubation, the supernatant was collected, aliquoted and stored at -80°C.

3. Cytokine Extraction From Snap-Frozen Placentae

Three snap frozen placentas were randomly chosen and pulverized using liquid nitrogen and a pre-cooled mortar and pestle. The tissue was then transferred to a pre-cooled 2 mL microcentrifuge tube and kept on ice. Tissue was suspended in 500 µL of Extraction Buffer and homogenized for 30 sec on ice using a Brinkman polytron homogenizer (setting 5). An additional 500 µL of buffer was added to each homogenate before the tubes were vortexed and centrifuged at 14,000 x *g* for 10 min at 4°C. Supernatants were collected, aliquoted and stored at –80°C. Placental cytokine levels were only determined at the four key time points.

4. Amniotic Fluid Preparation

Amniotic fluid samples were thawed on ice and centrifuged at 14,000 x *g* for 10 min at 4°C to remove debris prior to assay. Cytokine levels in amniotic fluid were only measured at the four key time points.

5. Enzyme Linked ImmunoSorbent Assay (ELISA)

The IL-10 and IFN–γ ELISAs were performed in 96-well, round bottom polyvinyl microtiter plates (Fisher Scientific, Pittsburg, PA). Wells were coated overnight at 4°C with 50 µL/well of 1 µg/mL capture antibody or isotype control (IgG1) in phosphate buffered saline (PBS). The plate was then washed twice with PBST, blocked for 30 minutes at room temperature with 100 µL/well of blocking buffer (20% (v/v) FBS in extraction buffer), and washed twice with PBST. Samples and standards were prepared in a separate plate using extraction buffer and PBS as the diluents for the placental extracts and amniotic fluid, respectively. A standard curve including a blank was generated using recombinant mouse IL-10 or IFN–γ and added in duplicate to the wells. Samples were assayed in quadruplicate in wells coated with capture antibody and in duplicate in wells coated with isotype control. 25 µL/well of samples and standards were incubated overnight at 4°C. Wells were emptied by inversion and incubated for 15 min at room temperature in a moist chamber with 20 µL/well of 5% H_2O_2 in 2nd Antibody Buffer Stock to remove endogenous peroxidases. Plates were then washed four times with PBST before incubation for 30 min at room temperature with 50 µL/well of 1 µg/mL biotinylated detection antibody in 2nd Antibody Buffer. Plates were

washed twice with PBST. Then, streptavidin/horseradish peroxidase conjugate diluted 1:5000 in 2nd Antibody Buffer (75 µL/well) was added for 30 min at room temperature. Plates were washed four times with PBST. Finally, 100 µL/well of TMB substrate solution (prepared just prior to use) was added and the colour reaction was allowed to develop in the dark for 20 min at room temperature. The enzyme/substrate reaction was stopped with 50 µL/well of a 1:20 dilution of H_2SO_4 and the optical density at $\lambda = 450$ with a reference wavelength of 570 nm was measured using an automated microplate spectrophotometer (UVmax Kinetic Microplate Reader, Molecular Devices Corporation, Menlo Park, CA). Results were expressed as pg IL-10/mL and pg IFN–γ/mL.

Characterization of Assay

Summarized in Table V-1 and V-2 are the accuracy and intra- and interassay coefficients of variation for each buffer used in the IFN–γ and IL-10 ELISAs, respectively. These parameters were calculated from two ELISAs performed with duplicate standard curves and quality controls in each. With respect to the limits of detection, the sensitivity of both the IFN–γ ELISA and IL-10 ELISA was 31.3 pg/mL.

Table V-1. IFN–γ ELISA Accuracy and Precision.

Concentration Added (pg/mL)	Concentration Observed (pg/mL)	Intra-Assay Coefficient of Variation (%)	Inter-Assay Coefficient of Variation (%)
Extraction Buffer			
200	247 ± 10	9.1	8.3
100	105 ± 7	15.1	16.2
50	55 ± 5	22.7	23.6

n=2 per group

Table V-2. IL-10 ELISA Accuracy and Precision.

Concentration Added (pg/mL)	Concentration Observed (pg/mL)	Intra-Assay Coefficient of Variation (%)	Inter-Assay Coefficient of Variation (%)
Extraction Buffer			
200	173 ± 11	3.9	12.5
100	101 ± 5	8.5	12.4
50	58 ± 2	5.3	6.6
PBS-Triton X-100			
200	211 ± 6	1.7	5.7
100	91 ± 3	1.3	8.7
50	40 ± 2	7.4	10.0
PBS			
200	174 ± 19	4.4	21.7
100	75 ± 12	5.0	37.8
50	59 ± 6	0	26.7

n=2 per group

Additional Assay Optimizations and Validations

Initial experiments were conducted to measure cytokines released in placental single cell cultures. However, the majority of samples measured were below the limits of detection in our assays despite the use of different cell concentrations ($1x10^6$ cells/mL, $5x10^6$ cells/mL) and incubation times (2 h, 24 h) as well as attempts to concentrate the samples using centrifugal filtration.

It was then decided that ELISAs would be developed in order to measure IL-10 and IFN–γ from frozen placentas which were readily available. This method would allow measurements of any cytokines that are present in the placentas at the time of tissue collection. Using a protocol and buffer (Extraction Buffer) suggested by Dr. Jeffrey Keelan,

University of Auckland, placentas were prepared and assayed in a basic ELISA for IFN–γ and IL-10 established by the Mosmann Laboratory. These preliminary assays revealed extremely high background readings even in control wells using a capture antibody that had no specificity for either cytokine but was identical in isotype (IgG1) to the IFN–γ and IL-10 capture antibodies.

The non-specific binding (high background) problem was ameliorated by the incorporation of several additional steps in the ELISA protocol. These additional steps were determined in the following sequence: (1) In subsequent IL-10 and IFN–γ ELISAs, it was shown that the addition of a blocking step using 20% FBS prior to incubation of samples and a H_2O_2 treatment step after sample incubation. The H_2O_2 irreversible inactivated and removed endogenous peroxidase (Movahedi *et al.*, 1999; Wu *et al.*, 1999) helped to decrease backgrounds. However, most of the signal observed was still background. (2) An overnight incubation of the samples at 4°C helped to further diminish the background. (3) Different types and percentages of serum in the Extraction Buffer and 2nd Antibody Buffer were assayed and the results suggested that 5% normal goat serum in both buffers provided the best signal to noise ratio. (4) The addition of 5% skim milk powder also decreased non-specific binding after an assay was performed using various concentrations of milk powder in the 2nd Antibody Buffer.

To validate the assays, samples were mixed with known concentrations of cytokines at the time samples and standards were added to the plate. Full recovery was observed. Lyophilization of the homogenates and reconstitution of the samples to half their original volume using milli-Q H_2O failed to show a doubling of the values measured. However, concentrating the sample also concentrated the salts within the sample which might have

affected the assay results and the antigen may have been lost. Furthermore, in samples which were lyophilized, there was very little or no cytokine present initially, so that concentrating the sample by a certain magnitude would not have increased the concentration accordingly. At the end of these optimizations and validations, it was decided that the modified ELISA protocol was finally suitable for measuring cytokines extracted from frozen placentas.

Failure to detect any IL-10 or IFN-γ in our assays suggested that perhaps all of the cytokines in the placentas were not being extracted. In the quest to optimize the ELISAs and diminish non-specific binding and the widely accepted general protocol used to extract proteins from frozen tissue, pre-homogenization spikes to look for recovery were not conducted. At the suggestion of Jeffrey Pollard (Albert Einstein College), a PBSX Buffer (1% Triton X-100, 1mM PMSF, 5 μg/mL Leupeptin, 0.15 /mL Aprotinin in PBS) was used as the extraction buffer. The addition of detergent would help to extract any membrane bound proteins. Attention was focussed on the IL-10 ELISA. Homogenates were prepared using either a polytron homogenizer or a Dounce homogenizer. Samples were mixed with various concentrations of recombinant cytokines prior to homogenization, post-homogenization or within the assay. Blanks consisting of buffer only were also prepared and spiked accordingly. Polytron homogenization resulted in recoveries ranging from 43-100% in blanks and 10-28.5% in samples. In contrast, dounce homogenization resulted in better recoveries for the blanks but failed to improve recovery values in samples. In blanks, there was full recovery of pre-homogenization spikes (97.1%) as well as within-assay spikes (94.1%). In contrast, sample recoveries for the pre-homogenization spikes and within-assay spikes were 23.9% and 37.7%, respectively. The accuracy and precision of these assays are summarized in Table V-1. Discussions over the results obtained suggested that something

within the homogenates may be degrading the cytokine (proteases), interfering with the ELISA antibodies (anti-Fc receptor antibodies) or binding to IL-10 (soluble receptors) to prevent its recognition by the ELISA antibodies. Thus, using this protocol, it would appear that the levels of these cytokines are at the limit of detection and the values obtained are greatly underestimated. Further experiments extracting IL-10 and IFN–γ from frozen placentas were not warranted.

In order to validate the IL-10 ELISA for amniotic fluid samples, test samples and buffers (PBS) were mixed with a known amount of recombinant IL-10 at the time of sample and standard incubation. In buffer, full recovery of the recombinant IL-10 was observed. However, IL-10 levels in test amniotic fluid showed greater than expected values (*i.e.* greater than 100% recovery). The reason for this amplification was unknown. Despite this anomaly, it was decided that the amniotic fluid IL-10 measurements should proceed, with the caveat that any values obtained may be over-estimations of the levels that actually exist *in vivo*.

6. Tumour Necrosis Factor (TNF) Bioassay

Cell Culture

L929-8 cells are subclones of the murine fibroblastoid cell line L929 and are sensitive to the cytolytic effects of TNF. L929-8 cells were the generous gift of the Larry J. Guilbert labortaory (Edmonton, Alberta). L929-8 cells were maintained in culture at 37°C, 5% CO_2 in Growth Media using sterile, 250 mL, 75 cm^2 Falcon flasks (Fisher Scientific, Nepean, Ontario). Cells were passaged two days prior to bioassay using trypsin-EDTA solution (3 mL) and were confluent at the time of the bioassay.

Bioassay

The TNF Bioassay was performed according to methods described previously (Branch *et al.*, 1991) with some minor modifications. Briefly, samples were pre-incubated with either a 1:4000 dilution of rabbit anti-TNF antibody or antibody buffer (Growth Media) at 37°C in 5% CO_2 for 2 h. Concurrently, TNF-sensitive L929-8 cells were added to the wells of a sterile, 96-well, flat-bottom polystyrene microtiter plate at a concentration of 5×10^4 cells per well in 50 µL of TNF Assay Medium supplemented with 2 µg/mL of actinomycin D. The plates were incubated at 37°C, 5% CO_2 for 2 h. A standard curve of recombinant mouse TNF (50 µL) was prepared in either extraction buffer for placental homogenates, or TNF assay media for amniotic fluid using doubling dilutions starting at 50 pg/mL. Standards were added in duplicate to the wells and undiluted samples were added in triplicate or quadruplicate. Once added to cells, the standard curve began at 25 pg/mL. The plates were cultured for an additional 18 h at 39°C (note higher temperature), 5% CO_2 to increase the sensitivity of the assay. The final dilution of rabbit anti-TNF antibody and the samples was 1:8000 and 1:4, respectively.

Following this incubation, the viability of the L929-8 cells was assessed using the neutral red vital stain as follows: 50 µL of a neutral red solution (warmed to room temperature prior to use) was added to each well and incubated at 37°C, 5% CO_2 for 2 h. The plate was emptied by inversion and rinsed once with 200 µL of room temperature PBS. Cells were then dissolved in 100 µL of a NaH_2PO_4 Alcohol Solution and the plate was gently mixed on a shaker for 5 min. Optical density at $\lambda = 570$ nm with a reference wavelength of 650 nm was determined using an automated microplate spectrophotometer (UVmax Kinetic

Microplate Reader, Molecular Devices Corporation, Menlo Park, CA). Results were expressed as pg TNF/mL.

Characterization of Assay

Bioassay accuracy and precision are summarized in Table V-3 for each of the buffers used. The sensitivity of the bioassay was 0.2 pg/mL. The TNF bioassay is sensitive to both TNF–α and TNF-β. However, L929-8 cells are unaffected by the presence of several other cytokines including IL-1 through IL-6, TGF-β and IFN–γ as detailed in Branch *et al.* (1991).

Table V-3. TNF Bioassay Accuracy and Precision.

Concentration Added (pg/mL)	Concentration Observed (pg/mL)	Intra-Assay Coefficient of Variation (%)	Inter-Assay Coefficient of Variation (%)
Extraction Buffer			
16	16.4 ± 0.5	9.2	9.3
4	4.3 ± 0.2	12.9	13.2
1	1.3 ± 0.1	15.0	17.4
TNF Assay Media			
16	17.2 ± 0.8	6.8	12.7
4	3.7 ± 0.3	4.6	23.2
1	1.0 ± 0.1	5.9	14.1

n=3-4 per group

Additional Assay Validations

Preliminary experiments were also conducted using the supernatants obtained from the placental single cell suspension cultures. However, no changes were evident in the term

birth time course, samples were at the limit of detection, and the problems experienced with the corresponding ELISAs rendered the continued use of this method undesirable.

In order to modify the TNF bioassay so that TNF levels could be measured from the homogenates of frozen placentas, standard curves were prepared using Extraction Buffer, PBSX or TNF Assay Media. It was determined that Extraction Buffer and TNF Assay Media were both compatible with the bioassay, but PBSX was incompatible regardless of the percentage of Triton X-100 present.

To determine the amount of cell death observed that was the consequence of TNF, antibodies against murine TNF were used to neutralize the TNF and prevent it from killing the cells. Thus, samples or extraction buffer alone were pre-incubated with antibody or antibody buffer as well as with or without a 4 pg/mL recombinant TNF addition. The results of this test are depicted in Figure V-1A. The same tests were repeated for amniotic fluid (Figure V-1B). To ensure that antibody was in excess, the standard curve was pre-incubated with either antibody or antibody buffer. Presence of the antibody abolished the standard curve. Thus, the antibody was in excess over the entire range of the standard curve. These results are shown in Figure V-2. We were unable to confirm the results of this bioassay with an ELISA because the values observed were below the limit of detection of an ELISA. Finally, to ensure that this method of pre-incubating the samples with or without anti-TNF antibody could detect differences in TNF levels over the entire range of the standard curve, a standard curve was incubated with or without antibody, the difference in optical density was graphed against TNF concentrations and statistical analysis (one-way ANOVA) was performed (Figure V-3). Each of the TNF concentrations studied were significantly different from each other and the blanks ($p<0.05$).

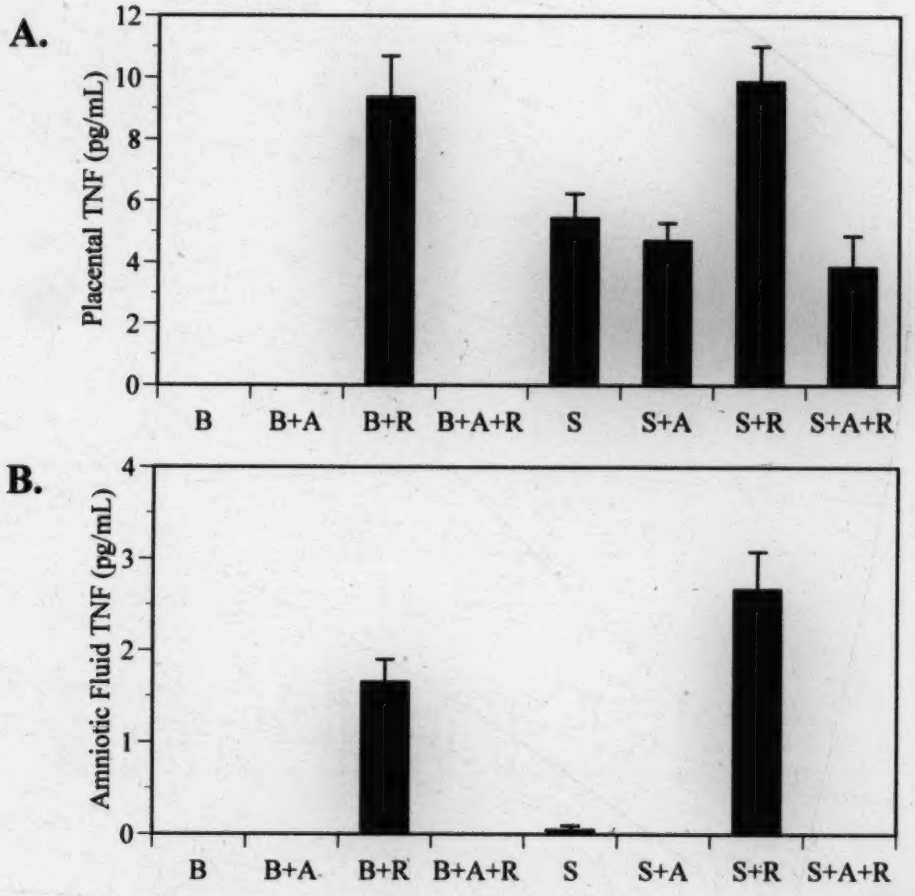

Figure V-1. TNF bioassay validation I. Buffers (B) and samples (S) were incubated with or without anti-TNF antibody (A) in the presence or absence of 4 pg/mL recombinant TNF (R) to determine whether the antibody could block the cell death due to TNF (see text for details).

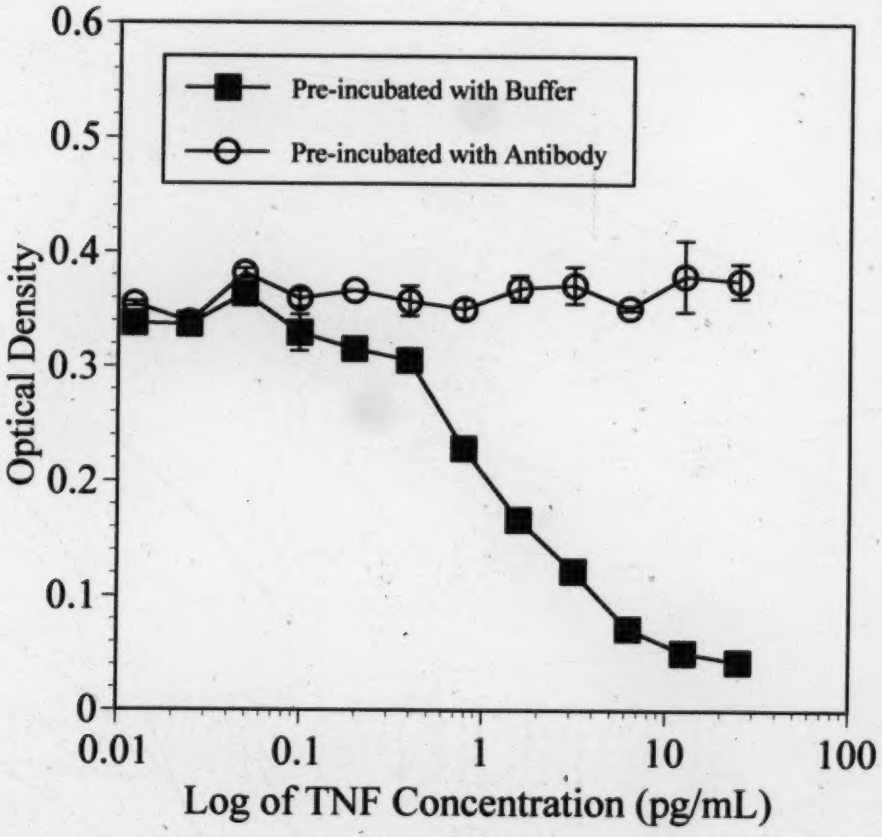

Figure V-2. TNF bioassay validation II. A standard curve was incubated with either anti-TNF antibody or antibody buffer in order to ensure that the anti-TNF antibody was able to neutralize TNF over the entire range of TNF concentrations present in the standard curve and was therefore in excess.

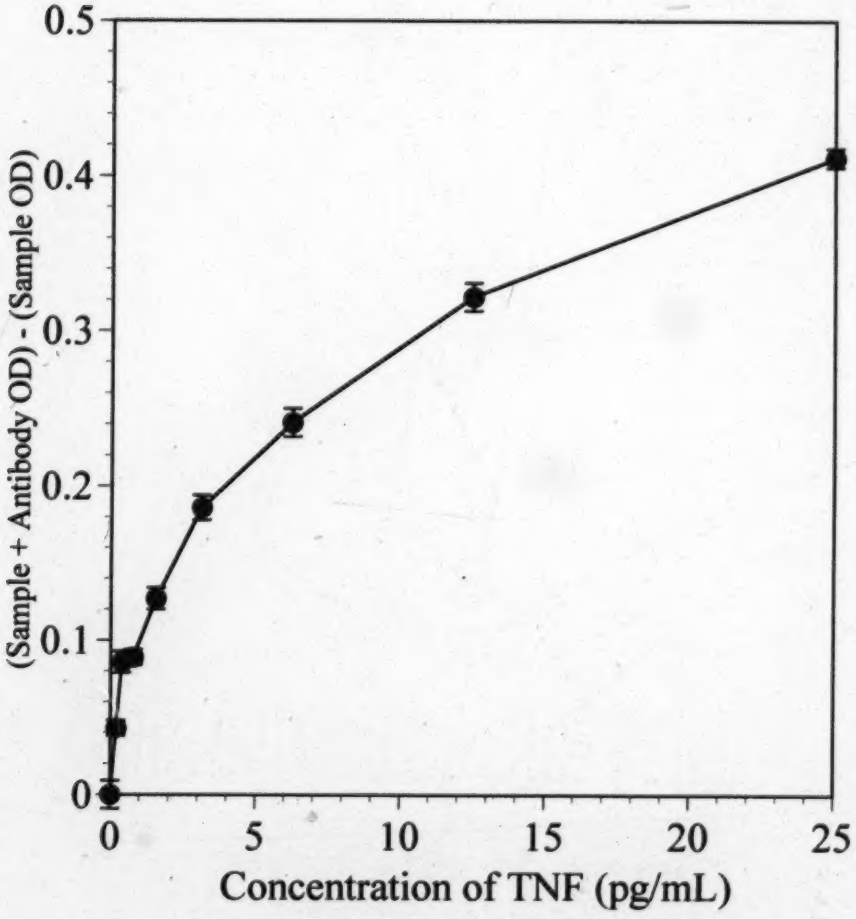

Figure V-3. TNF bioassay validation III. The difference in optical density between various concentrations of TNF pre-incubated with anti-TNF antibody or antibody buffer was determined to ensure that use of the anti-TNF antibody to detect the cell death due to TNF was valid over the range of TNF concentrations that could be measured in the bioassay.

7. Statistical Analysis

All results are expressed as the mean ± SEM. In order to detect changes with advancing gestational age, a one-way ANOVA (SigmaStat version 2.0, SPSS Inc.) was conducted for the untreated control time course with post-hoc analyses by Tukey's test as required. Since only two time points (GD 16.0 and Preterm Birth) were examined in the ethanol-treated time course, a Student's t-test was used to examine the effect of gestational age on cytokine levels. Further analyses with Student's t-tests were utilized to examine differences between the untreated (control) and ethanol-treated (experimental) groups at key time points. These included comparisons between term birth and preterm birth groups as well as comparisons between preterm birth and its gestational age-matched control. A statistical level of significance of $p \leq 0.05$ was chosen.

C. RESULTS

1. Placental Interferon–γ

IFN–γ was only measured from placentas since amniotic fluid samples were limited in quantity and reserved for the IL-10 and TNF assays. Table V-4 shows the data obtained at the four key time points in this study. IFN–γ was not detectable at any of these points.

Table V-4. Placental IFN–γ Concentrations at the Key Gestational Time Points in Untreated Controls and Ethanol-Treated Mice.

Mouse Group	Gestational Age		
	GD 16.00	GD 17.75	GD 19.1
Placental IFN-γ (ng/mL)			
Untreated Control	ND	ND	ND
Ethanol Treated	ND	ND	-

ND = Not Detectable (i.e. below assay sensitivity limit of 31.3 pg/mL), n=3 per group

2. Placental and Amniotic Fluid Interleukin-10

Placental and amniotic fluid IL-10 concentrations are summarized in Table V-5. Placental IL-10 levels were not detectable. In contrast, amniotic fluid IL-10 concentrations were consistently low and did not change regardless of gestational age or treatment group.

Table V-5. Placental and Amniotic Fluid IL-10 Concentrations at the Key Gestational Time Points in Untreated Controls and Ethanol-Treated Mice.

Mouse Group	Gestational Age		
	GD 16.00	GD 17.75	GD 19.1
Placental IL-10 (pg/mL)			
Untreated Control	ND	ND	ND
Ethanol Treated	ND	ND	-
Amniotic Fluid IL-10 (pg/mL)			
Untreated Control	37 ± 2	47 ± 4	54 ± 11
Ethanol-Treated	37 ± 2	43 ± 1	-

ND = Not detectable (i.e. below assay sensitivity limit of 31.3 pg/mL), n=3 per group

3. Placental and Amniotic Fluid Tumour Necrosis Factor

TNF from amniotic fluid was also not detectable with increasing gestation or treatment group and this data is available in Table V-6. In contrast, there was a significant effect of gestation on placental TNF levels ($F(2,31)=7.744$, $p=0.002$) and post-hoc analysis with Tukey's test revealed that placental TNF levels were significantly elevated during term birth when compared to GD 16.00 ($p<0.05$). However, there was no significant effect of time on placental TNF levels in the ethanol-treated mice ($t_{20}=1.06$, $p=0.30$). These changes are reflected in Figure V-4. As shown in Figure V-5, placental TNF levels were higher during term birth than preterm birth but this difference did not achieve statistical significance ($t_{19}=1.54$, $p=0.14$). Finally, when an analysis of the effect of ethanol on placental TNF levels on GD 17.75 (Figure V-6) was performed, no statistically significant difference was detected ($t_{20}=0.49$, $p=0.63$).

Table V-6. Amniotic Fluid TNF Concentrations at Key Gestational Time Points in Untreated Controls and Ethanol-Treated Mice.

Mouse Group	Gestational Age		
	GD 16.00	GD 17.75	GD 19.1
Amniotic Fluid TNF (pg/mL)			
Untreated Control	ND	ND	ND
Ethanol Treated	ND	ND	-

ND = Not Detectable (i.e. below assay sensitivity limit of 0.2 pg/mL), n=3 per group

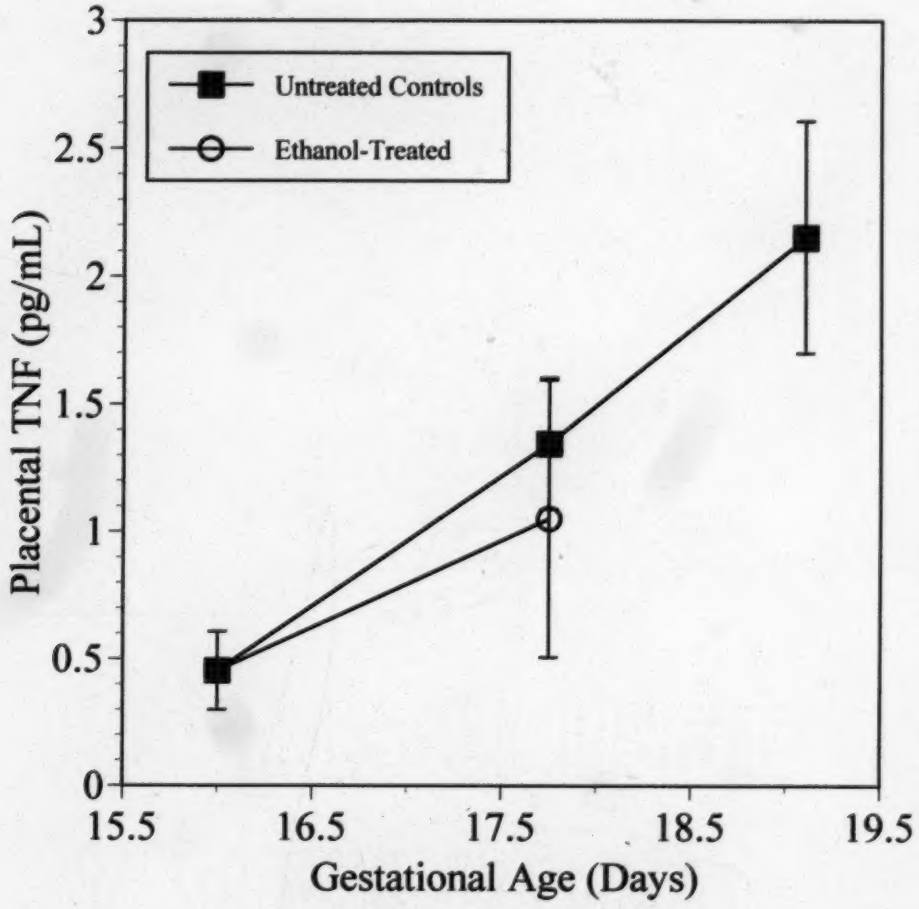

Figure V-4. The effect of gestational age on placental TNF concentrations (mean ± SEM) in ethanol-treated (n=11 per group) and untreated controls (n=10-11 per group). A gestational age-related difference when compared to GD 16.0 ($p \leq 0.05$) was observed in the untreated controls. No change with increasing gestational age was noted in the ethanol-treated mice ($p > 0.05$).

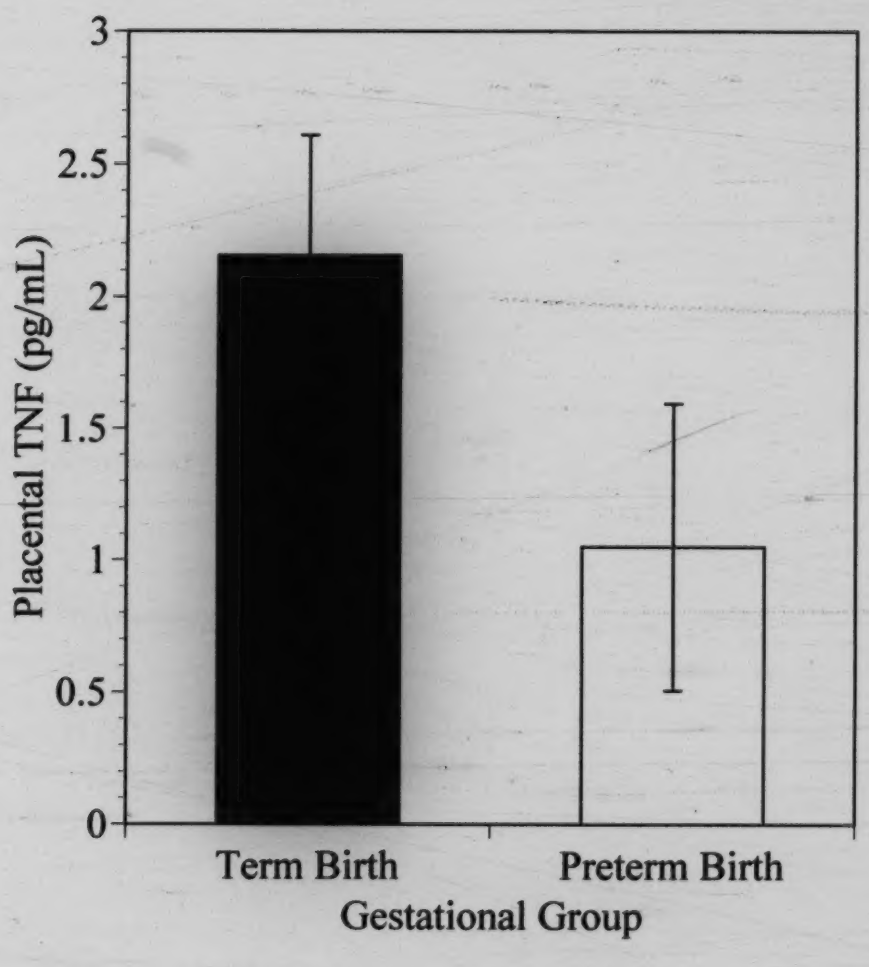

Figure V-5. The effect of ethanol on placental TNF concentrations (mean ± SEM) during parturition at term (n=10) and preterm (n=11). There was no significant difference ($p>0.05$).

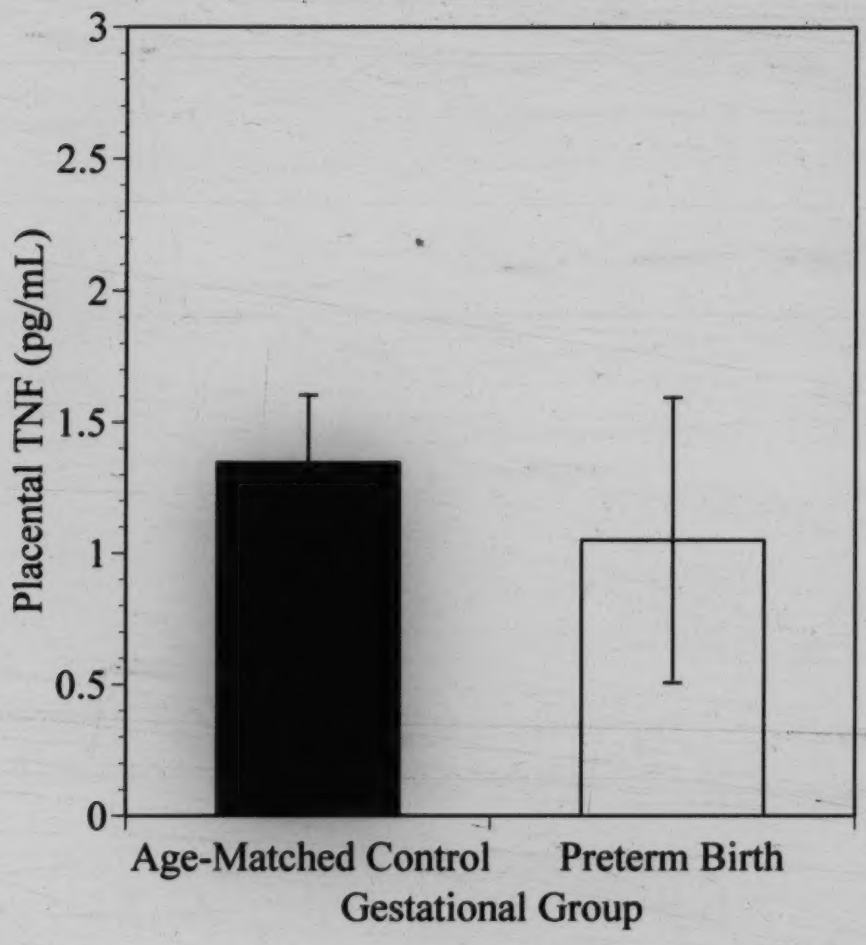

Figure V-6. The effect of ethanol on placental TNF concentrations (mean ± SEM) on GD 17.75 in untreated controls (n=11) and ethanol-treated mice (n=11). There was no significant difference ($p>0.05$).

D. DISCUSSION

The results of these experiments are inconclusive with respect to the effect of gestational age and ethanol on IFN-γ and IL-10 levels since the levels of these cytokines were low or not detectable in our mice. The inability to detect IFN-γ is in agreement with Lin *et al.* (1993) who were unable to detect placental IFN–γ late in gestation, but it is in contradiction to the high placental IFN–γ mRNA reported by Delassus *et al.* (1994). Placental IL-10 was not detectable late in gestation or during birth in our mice contrary to the reports of Lin *et al.* (1993) and Delassus *et al.* (1994) who were able to detect IL-10 protein and mRNA, albeit at low levels late in gestation. However, amniotic fluid IL-10 was detected but did not change with gestation or ethanol treatment. Dudley *et al.* (1997) also reported no change in amniotic fluid IL-10 levels in women at term or preterm. The majority of the measurements reported by Dudley *et al.* (1997), were in the 100-200 pg/mL range which is similar to our observations. The relevance of amniotic fluid IL-10 is unknown although it may play a role in fetal growth (Rijhsinghani *et al.*, 1997). Fortunato *et al.* (1997) have shown that IL-10 is not effective in altering the production of TNF-α when administered alone, but is effective in response to co-challenge by LPS. In contrast, TGF-β on its own was able to reduce TNF-α protein, but had no effect on LPS-induced TNF release by fetal membrane explants obtained by caesarean section.

The differences observed may also be attributed to mouse strain-specific effects, different antibodies and differences in sources of samples. For example, cytokines were measured from frozen placentas in this study, but other investigators used supernatants from decidual explants or placental single cell suspensions (Dudley *et al.*, 1993; Lin *et al.*, 1993).

The *in vitro* release of cytokines may not be an accurate reflection of *in vivo* conditions. Furthermore, several studies have shown evidence of an involvement of pro-inflammatory cytokines in bacteria- and LPS-induced preterm birth in mice. It is not surprising that cytokines were readily detectable in the presence of potent stimulators (*i.e.* bacteria and LPS) of cytokine production.

The assays described in this chapter have been specifically and thoroughly optimized and validated for the measurement of IL-10 and IFN-γ in amniotic fluid and frozen placentas. Furthermore, the sensitivity of these assays are identical to, if not better than, commercially available kits. Our inability to detect these cytokines despite equivalent assay sensitivities casts doubts on the cytokine values reported in the literature.

Tumour necrosis factor was not detected in amniotic fluid. In contrast, placental TNF was elevated during term birth, but not during preterm birth. De *et al.* (1992) reported a lack of TNF-α mRNA or bioactivity in the placenta late in gestation, but there was an increase in TNF-α bioactivity in the uterus on the day before parturition. However, this increase in bioactivity was not mirrored by mRNA levels since mRNA levels actually decreased at this time. No explanation was offered for this contradiction. These data support our hypothesis that there is an enhancement of pro-inflammatory cytokine secretion during parturition and suggests a potential role for TNF in the physiology of term birth. However, it could not be determined whether this increase in placental TNF was a cause or consequence of labour. In order to further dissect the role of placental TNF in term birth, measurements need to be made at additional time points. If the TNF increase precedes term birth, this would be suggestive of a causative role for TNF in parturition.

Elevations in TNF-α preceded infection-associated preterm birth as illustrated by Hirsch *et al.* (1999) who used snap-frozen uteri and placentas to show a time-dependent increase in uterine TNF-α in response to *E. coli* infection. They did not examine changes associated with term birth. The quantities of TNF-α measured by Hirsch *et al.* (1999) in their control samples were higher than quantities observed in this thesis. This may be a consequence of the different mouse strains, different extraction techniques and assay techniques employed. For example, neither protease inhibitors nor hydrogen peroxide (to remove endogenous peroxidases) were used by Hirsch *et al.* (1999). Furthermore, Hirsch *et al.* (1999) validated their assay through the full recovery of exogenous additions added to the tissue homogenates, but they failed to specify whether these additions were made prior to or after homogenization. However, the significant difference in TNF levels observed in this thesis was in the pg/mL range and the biological significance at this extremely low concentration may be relevant but is questionable nonetheless. As a point of reference, the equilibrium constant for the TNF receptors is approximately in the 1-10 ng/mL range (Tsujimoto *et al.*, 1986; Corti *et al.*, 1994). Furthermore, *in vitro* studies typically utilize concentrations of TNF-α ranging from 50-100 ng/mL in order to elicit the desired cellular responses (Dudley *et al.*, 1993; Spaziani *et al.*, 1998)

Effect of Alcohol

There is abundant evidence that alcohol can affect cytokine networks (reviewed in Watson *et al.*, 1994; Deaciuc, 1997). However, the pattern of ethanol consumption can have strikingly different effects. Furthermore, there has been very little investigation into the immunomodulatory effects of acute ethanol during pregnancy and parturition.

In non-pregnant humans, studies on the effects of ethanol consumption on cytokine production are derived primarily from observations that individuals who consume alcohol are more susceptible to infection. Consequently, these studies expose subjects to acute ethanol, and then examine the ability of the cells, tissues or subjects to respond to infectious stimuli in the form of bacteria or bacterial products such as endotoxin (reviewed in Friedman, 1998). The general consensus which arises from these studies indicates a promotion of TH2 and anti-inflammatory cytokine production and a suppression of TH1 and pro-inflammatory cytokines in response to the infectious insult (Szabo *et al.*, 1996, Szabo, 1998; Arbabi *et al.*, 1999). The same pattern is observed in mice since acute ethanol decreased TNF and neutrophil recruitment in response to bacterial infection in mice (D'Souza *et al.*, 1995). There is very little known on the effect of ethanol on basal production of cytokines.

The non-pregnant binge-drinking model in mouse described earlier (see Chapter 2) has clearly been associated with ethanol-induced changes in immune function. Through a series of experiments, acute ethanol exposure exerted its immunomodulatory effects via either a glucocorticoid dependent or independent pathway which could involve other mediators or direct effects of ethanol itself. Enhanced endogenous glucocorticoid levels in these mice were responsible for (1) thymic atrophy by depletion of immature thymocytes (CD4+CD8+) through apoptosis (Han *et al.*, 1993) and (2) the suppression of splenic IL-1β, IL-2 and IL-4 mRNA and a primary humoral response to sheep red blood cells. Glucocorticoids and direct effects of ethanol were jointly responsible for decreased basal and induced splenic NK activity (Wu and Pruett, 1996a and 1996b; Wu and Pruett, 1997). Vinson *et al.* (1998) showed that ethanol exposure decreased the neutrophil accumulation

and TNF production due to intra-peritoneal bacterial challenge. This occurred in a glucocorticoid-independent fashion. Since ethanol affects immune function in these mice, there may be a role for cytokines but the effects of alcohol on basal cytokine production have not been reported.

The absence of an increase in TNF in ethanol-induced preterm birth suggest that the immunosuppressive effect of ethanol holds true. This suggests that cytokines are not indispensible for birth but may facilitate the process both at term and preterm. In other words, the cytokine network may represent a redundant system that can be elicited in certain instances such as infection, but is not an absolute prerequisite for pregnancy termination. Although knockout mice which are deficient in the production of various cytokines are available, they have not been studied in the context of birth. Further investigation is required.

Future Studies

During the course of these experiments, many technical difficulties were encountered. While working through these problems, it became apparent why there were so few studies looking at basal tissue levels of cytokines. Concentrations were very low in the first place and something in the frozen placental homogenates interfered with the ability of cytokines to be bound in the sandwich ELISA. Furthermore, additional cytokines should be examined to obtain a more thorough representation of each subset. Extractions should be attempted from freshly collected tissue to better represent the conditions, *in vivo*, and avoid any problems associated with snap freezing. Also, cytokine content of other tissues such as uterus and fetal membranes should be examined and the full time course should be evaluated to obtain a more comprehensive picture and decrease the probability of missed changes.

These studies focussed on the measurement of the cytokine protein because the protein represented the biologically active moiety. However, future studies may choose to evaluate cytokine expression at the mRNA level. Since ELISAs only measure the protein content, the establishment of additional bioassays could determine biologically active protein as well as enhance sensitivity. Finally, three randomly chosen placentas were used in the assays, but they may not be representative of an entire litter. Cytokine measurements in the literature rely heavily upon ELISA kits, as well as samples obtained from cell culture supernatants, and there are very few studies which have examined the levels of cytokines from frozen tissue. Perhaps a part of this deficiency is due to the same difficulties encountered during the establishment of these assays in our laboratory. However, ELISAs need to be optimized specifically for the tissue from which cytokines are being measured. It is always possible to obtain a signal in ELISAs, but it is necessary to ensure that the signal is real and not background (i.e. like using H_2O_2 to remove endogenous peroxidases in our tissues which would give falsely elevated measurements). Finally, unstimulated basal cytokines levels and the effect of ethanol need to be established.

E. CONCLUSION

In conclusion, the data suggest a potential role for TNF in term birth. The elevated placental TNF levels may be construed as a shift towards pro-inflammatory cytokine release during term birth, but this shift was absent in ethanol-induced preterm birth. However, the experiments described herein are far from conclusive and further investigation is indicated. The inability to detect IL-10 and IFN-γ renders it impossible to address the hypothesis stated

at the beginning of the chapter. This is one of the first times that ELISAs and bioassays have been modified to measure cytokines from frozen tissues. Furthermore, the data presented herein represent a novel attempt to address the involvement of the TH1/TH2 and pro-/anti-inflammatory paradigms in the same batch of mice during term birth and ethanol-induced preterm birth.

CHAPTER VI. CONCLUSIONS

A. MEDIATOR INTERACTIONS

Throughout this thesis, the role of steroids, prostaglandins, TH1/TH2 and pro-/anti-inflammatory cytokines in rodent and human parturition have been discussed separately. However, these mediators interact extensively *in vivo* so that alterations in one cascade will undoubtedly influence the other pathways. The regulation of the delicate balance between these mediators and their activities is complex and is the focus of current research.

Steroids and Prostaglandins

On the one hand, progesterone and estrogen regulate prostaglandin bioactivity as part of their respective roles in maintaining uterine quiescence and uterine activation. In support of this conjecture, Abel and Baird (1980) have demonstrated that progesterone inhibits $PGF_{2\alpha}$ production and estradiol stimulates prostaglandin production in human non-pregnant endometrial tissues *in vitro.* In pseudopregnant rats, the *in vitro* release of PGE and $PGF_{2\alpha}$ from whole uterus was low when endogenous progesterone levels were high and increased when progesterone levels were low (Motta *et al.*, 1995).

Elevated prostaglandin bioactivity is achieved through several mechanisms including increased synthesis and decreased metabolism as well as an increase in the sensitivity of tissues to prostaglandins through the up-regulation of appropriate receptors. The association between high progesterone levels and low prostaglandin levels may be attributed to the

inhibition of PGHS-2 by progesterone (Hedin, 1997). Alternatively, Patel *et al.* (1999) have shown that progesterone increases PGDH (enzyme for prostaglandin catabolism) activity in placenta and chorion *in vitro.* At term, trilostane (3β-HSD inhibitor) decreased PGDH mRNA relative abundance in both tissues, and co-incubation with progesterone reversed this. Thus, any prostaglandins that are produced in intrauterine tissues and are in contact with chorion or placenta trophoblasts are efficiently inactivated through the actions of PGDH (Challis *et al.*, 1999). Furthermore, progesterone limits the contractile activity of prostaglandins in mouse uteri by up-regulating the mRNA for relaxation-inducing receptors such as EP_2 (Lim and Dey, 1997). Estradiol exhibits effects opposite to those of progesterone in each case. Thus, estrogens and progesterone can influence prostaglandin bioactivity by regulating production and clearance.

Alternatively, prostaglandins may exert their influence by modifying the steroid environment through the initiation and progression of functional luteolysis. Functional luteolysis refers to the specific loss of steroidogenic capacity during regression of the corpus luteum (McCracken *et al.*, 1999). In rats, $PGF_{2\alpha}$ treatment can induce preterm birth, and it is believed that the luteolytic action of $PGF_{2\alpha}$ is responsible because the induction of preterm birth can be prevented by concurrent progesterone replacement (Alexandrova and Soloff, 1980). Strong evidence for a role for prostaglandins in the induction of luteolysis in mice arises from studies conducted using targeted gene disruption in mice. Mice deficient in the FP receptor (Sugimoto *et al.*, 1997) or PGHS-1 (Gross *et al.*, 1998) were able to establish and maintain pregnancy, but exhibited gestational lengths which extended beyond term. In both cases, elevated serum progesterone levels implicated an impairment of luteolysis as the impetus behind prolonged gestational lengths. Luteolytic deficiencies in these strains were

attributed to loss of $PGF_{2\alpha}$ luteolytic bioactivity. In the PGHS-1 knockouts, inadequate levels of $PGF_{2\alpha}$ were present since PGHS-1 was responsible for the majority of uterine $PGF_{2\alpha}$ production (Gross *et al.*, 1998) and in the FP knockouts, $PGF_{2\alpha}$ was unable to interact with its receptor. The studies reported by Sugimoto *et al.* (1997), however, failed to address why these mice possessed normal estrous cycles despite their inability to initiate luteolysis. This raises the possibility that luteolysis during pregnancy is different than luteolysis in non-pregnant mice.

Cytokines and Steroids

Siiteri *et al.* (1977) first suggested that progesterone exerts immunosuppressive and anti-inflammatory effects. While this may be mediated by the progesterone-associated suppression of prostaglandins, progesterone can also influence the cytokine milieu. In the presence of progesterone at concentrations present in the placenta (3 μg/g placenta), the development of TH2 cells in culture is favoured (Piccinni *et al.*, 1995). Lymphocytes possessing progesterone receptors will release a 34 kDa protein, the progesterone induced blocking factor (PIBF), which causes activated lymphocytes to up-regulate production of TH2 cytokines (IL-10 and IL-4) (Szereday *et al.*, 1997), inhibits NK cell activity and prevents fetal resorptions in mice (Szekeres-Bartho *et al.*, 1996).

The effects of estrogen and progesterone are often antagonistic. In non-pregnant, ovariectomized mice, estrogen exhibits pro-inflammatory effects which promote the influx of neutrophils and macrophages into the uterus, while progesterone administered concurrently antagonized this action (Tibbetts *et al.*, 1999). In order to facilitate the recruitment of immune cells, estradiol may stimulate the expression of adhesion molecules

and this has been shown in cultured human umbilical vein endothelial cells obtained after term labour (Winkler *et al.*, 1997). Estradiol also has the capacity to stimulate TNF expression in mouse myometrium, and this expression was inhibited by progesterone (Roby and Hunt, 1995).

Steroid effects on cytokine production may be mediated by the transcription factor, NF-κB. Cytokine or steroid-induced elevations in NF-κB culminates in the up-regulation of pro-inflammatory cytokine synthesis in human placental HTR-8 cells *in vitro* (Leslie and Feldman, 1999). Conversely, progesterone decreases LPS-induced TNF-α mRNA and protein in mouse macrophages by increasing IκB-α (an inhibitor of NF-κB) and thereby decreasing the NF-κB-induced transcription of TNF (Miller and Hunt, 1998).

The interaction between cytokines and steroids is bi-directional. Cytokines may regulate steroidogenesis in the placenta (Feinberg *et al.*, 1994). Human placental cytotrophoblasts in culture with androstenedione responded to IL-1 treatment by increasing the aromatization of androstenedione to estrogen (Nestler, 1993). If endogenous IL-1 was then removed by antibody incubation, this conversion to estrogen decreased (Nestler, 1993). It has also been suggested that pro-inflammatory cytokines induced by LPS have an anti-gonadotropic effect (Fidel *et al.*, 1998).

Prostaglandins and Cytokines

Parturition is an instance when inflammatory processes which are normally associated with pathophysiological conditions, effects normal physiological change. In animal models of preterm labour, increases in cytokines are important for the induction of prostaglandin synthesis as well as the synthesis of additional cytokines. Decidual explants

from pregnant mice were able to respond to endotoxin and pro-inflammatory cytokines by increasing PGE_2 and IL-6 production (Dudley *et al.*, 1993). Pro-inflammatory cytokines can also increase PGE_2 and $PGF_{2\alpha}$ output by human intrauterine cells *in vitro* (Brown *et al.*, 1998b). The stimulation of *in vitro* cytokine production by LPS and cytokines is an important reflection of the ability of these tissues to respond to infectious insults such as bacteria and bacterial endotoxins. This suggests that cytokine synthesis may be recruited into the parturition cascade in certain circumstances. Cytokines may also be involved in the induction of the prostaglandins which are responsible for luteolysis. In support of this idea, LPS-induced preterm birth in mice is associated with a fall in progesterone levels (Fidel *et al.*, 1998). This suggested that LPS treatment elicited pro-inflammatory cytokine secretion which then increased the production of luteolytic prostaglandins. Endotoxin or pro-inflammatory cytokines can stimulate PLA_2 or PGHS-2 in human intrauterine tissues (Mitchell *et al.*, 1993c; Nguyen *et al.*, 1994; Hansen *et al.*, 1999; Pomini and Challis, 1999). These data and the data above suggest that cytokines increase prostaglandin bioactivity by increasing prostaglandin production.

Alternatively, endotoxin and pro-inflammatory cytokines can also enhance prostaglandin bioactivity by diminishing PGDH activity and decreasing catabolism (Brown *et al.*, 1998a; Challis *et al.*, 1999; Pomini and Challis, 1999). In addition, the expression of EP_1 receptors can be up-regulated by pro-inflammatory cytokines (Spaziani *et al.*, 1997). Anti-inflammatory cytokines such as IL-10, on the other hand, can block the effects of these pro-inflammatory cytokines (Pomini and Challis, 1999).

There is evidence that PGE may have both (1) pro-inflammatory effects by virtue of its ability to recruit immune cells into tissues and (2) anti-inflammatory effects through its

inhibition of NK cell cytotoxicity, inhibition of TH1 cells and stimulation of TH2 cells (Kelly, 1996). However, these effects are dependant upon the cellular distribution and local metabolism of PGE according to Kelly (1996).

An overall schematic of these potential bi-directional interactions is summarized in Figure VI-1 below. The unknown interactions (denoted by question marks) in the schematic may be the focus of future studies.

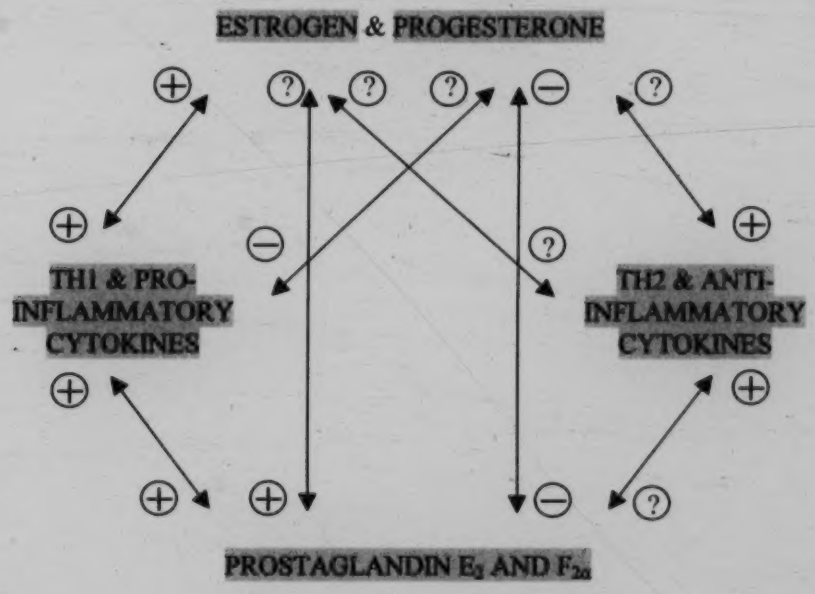

Figure VI-1. Summary of potential mediator interactions during term birth in mice.

Effect of Ethanol

The effects of ethanol are dependant upon the pattern of ethanol exposure which may be chronic (long-term) or acute (short-term). This thesis focuses on the effects of a single bolus of ethanol. Ethanol can disrupt the cAMP and PLC signalling pathways by altering the intracellular localization of PKA and PKC (Gordon *et al.*, 1998). Ethanol also increases

adenylate cyclase activity and cAMP accumulation (see Rabin, 1993). A review by Hoek and Kholodenko (1998) suggested that ethanol enhances hormone-stimulated cAMP production but further studies need to be done on the effects of ethanol in the context of pregnancy.

The results obtained from this thesis suggest that ethanol exposure skips several steps in the cascade of events leading to parturition, but still arrives at the same end result of birth. Although ethanol-induced preterm birth was not associated with an elevation of plasma 17β-estradiol levels, an overall increase in the E:P ratio was still achieved. Similarly, $PGF_{2\alpha}$ bioactivity was enhanced during ethanol-induced preterm birth, not by increasing levels, but increasing the sensitivity of target tissues to $PGF_{2\alpha}$ by increasing the FP receptor. Finally, ethanol administration does not require TNF, a representative of pro-inflammatory cytokines.

B. FINAL CONCLUSIONS

The available evidence suggests that the intricate network of cascades which culminate in the phenomenon of birth is exquisitely regulated. At term, there is a complex interaction of these mechanisms to bring about efficient labour and delivery. Preterm birth triggered by independent stimuli may preferentially activate some of these mechanisms and bypass others but still achieve the same end result.

While it is important to dissect out the specific roles of the mediators, it is still their interaction *in vivo* which is of paramount importance. These studies are innovative because several mediators are being measured in a concerted manner in the same mice. Furthermore, both term birth and preterm birth have been examined and fairly extensive chronological profiles of steroid hormones, prostaglandins, IFN-γ, IL-10 and TNF have been obtained. The

results of the experiments presented in this thesis suggest that the mechanisms underlying term parturition are different than those underlying ethanol-induced preterm parturition in the C57BL/6J mouse. Thus, the overall hypothesis presented in Chapter 1 has been refuted. Studying term birth or preterm birth in isolation is not sufficient to gain a comprehensive understanding of the mechanisms. By examining both, it is possible to identify the similarities and differences and hopefully, exploit the differences in future intervention studies.

The predominant focus of this thesis was on the development and establishment of various techniques for the measurement of steroid hormones, prostaglandins, IFN-γ, IL-10 and TNF from mouse tissue. As a consequence of the work summarized by this thesis, several techniques have been established in our laboratory. Furthermore, several questions have been raised concerning the validity of other methodologies commonly used in other studies. Thus, a solid foundation has been established for exciting future studies exploring the events preceding parturition in the mouse.

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